Proceedings
of the
Society
for
Experimental Biology and Medicine

Vol. 77 August, 1951 No. 4

Effect of Cortisone upon Passive Anaphylaxis in the Guinea Pig.* (18858)

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The mechanism of action of cortisone upon diseases of allergy or hypersensitivity(1) is unknown, though the marked effect of this hormone upon tissue proliferation certainly contributes significantly to this suppressive or masking effect(2-4). The anaphylactic response of sensitized tissues, however, is independent of an inflammatory reaction or cellular proliferation, and though quantitative studies of anaphylaxis in animals sensitized by active immunization are unsatisfactory because of the tremendous variation in the ability of individual animals to produce antibodies to a similar stimulus and our inability to measure the total antibody produced by or present in an organism at a given moment, Kabat and his coworkers(5-7) have shown that quantitative studies of passive anaphylaxis are very reproducible. Although several investigators(8-13) have obtained varying effects with cortisone in anaphylaxis, the application of these quantitative technics (5-7) with challenge *in vitro* by the method of Schultz(14) and Dale(15) has brought forth further significant data.

Methods. Virginal female guinea pigs weighing 250 ± 50 g and on *ad lib.* feeding were used in all experiments. Sensitizing injections were given intravenously using the veins on the ventral surface of the hind legs. The antibody used was in the form of rabbit antisera to type III pneumococci, calibrated by the method of Heidelberger and Kendall

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* Supported in part by a Research Grant-in-Aid, National Institutes of Health, U. S. Public Health Service.

† Postdoctorate Research Fellow, National Institutes of Health, U. S. Public Health Service.

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14. Schultz, W. H., *J. Pharm. and Exp. Therap.*, 1910, v1, 549.

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(16). All animals were sacrificed by a blow to the head 48 hours following sensitization. The uterine and bowel strips were mounted in a 70 ml bath of Ringer-Locke's solution according to the technic of Dale(15). The experiments were carried out immediately following the death of the animal, except for occasional experiments in which the tissues were chilled prior to use. One uterine horn and one bowel strip were usually mounted together in the same bath and recorded simultaneously, thereby serving as controls for one another. The bowel strips were obtained from the ileum and jejunum of the guinea pigs, and no bowel strip was considered to be insensitive until at least three strips from different sections of the lower small bowel gave a negative response. All bowel and uterine strips showing a negative reaction were tested for viability with histamine at the conclusion of each experiment. The antigen, purified Specific Soluble Substance III, was shown to be non-irritating to normal muscle strips on many occasions. The average challenging dose of 20 γ was added to the bath with a pipette. The pipette was inserted to the bottom of the bath and then slowly withdrawn as the antigen was allowed to flow into the bath, thus facilitating better distribution. All contractions were recorded on revolving smoked drums. The injections of cortisone or control solutions were started two days prior to sensitization and continued until sacrifice of the animals, 2 days after sensitization. The cortisone‡ treated animals were injected with 2.5 mg of the hormone in the deep subcutaneous tissues morning and night except during some of the early experiments when only one injection each day of 5 mg was given. In 9 animals cortisone was not started until 18 hours after the sensitizing injection.

TABLE I. Effect of Cortisone upon Passive Sensitization of Guinea Pig Uterine Muscle.

Antibody N given	Controls	Cortisone treated, 96 hr	Cortisone treated, 30 hr
25 γ			
Contracting	17	9	6
Non-contracting	0	0	0
50 γ			
Contracting	4	11	2
Non-contracting	0	0	0
100 γ			
Contracting	0	1	1
Non-contracting	0	0	0
Totals			
Contracting	21	21	9
Non-contracting	0	0	0

TABLE II. Effect of Cortisone upon Passive Sensitization of Guinea Pig Small Bowel.

Antibody N given	Controls	Cortisone treated, 96 hr	Cortisone treated, 30 hr
25 γ			
Contracting	15	2	5
Non-contracting	2	7	1
50 γ			
Contracting	4	4	2
Non-contracting	0	7	0
100 γ			
Contracting	0	1	1
Non-contracting	0	0	0
Totals			
Contracting*	19	7	8
Non-contracting*	2	14	1

* Statistical probability of this being chance variation less than 1 in 1000, as determined by the Chi square test.

Results. Table I summarizes the effect of cortisone upon the passive sensitization of uterine smooth muscle as measured by the Schultz-Dale technic. In no animal was sensitization affected by cortisone.

Table II summarizes the rather striking effect of cortisone upon the passive sensitization of guinea pig small bowel. Of the 21 controls, 19 showed good sensitization, while only 2 failed to demonstrate sensitivity. In the cortisone treated group, two-thirds of the cases(14) showed an absence of sensitization and only one-third(7) demonstrated definite sensitivity. In 8 of 9 animals treated with cortisone 18 hours after the sensitizing injection had been given, the bowel strips demonstrated good responses to the challenging antigen.

Five of the control animals were injected twice daily with the vehicle used for suspending the cortisone solutions and 6 others simultaneously received injections of normal saline. All of these animals showed sensitization except one in the normal saline group. Six

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16. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1937, v65, 647.

‡ Merck brand Cortone.

other animals were given cortisone for 96 hours, but no sensitizing injection. They showed no abnormal reactions to the challenging antigen.

Both animals in the control series showing no bowel sensitization received a sensitizing dose of 25 γ of antibody N while 5 of the 7 cortisone-treated animals showing definite sensitization received at least twice this dosage of antibody, an amount never failing to sensitize control animals. Although exactly comparable amounts of antibody were not used in all experiments, it will be noted that the variations were in the direction of less antibody injected into the controls and more antibody given the cortisone-treated animals. Such variation should add further weight to the recorded figures. The amounts of antigen used for challenging were also varied in some instances, again in such a manner as to make the experimental results more significant.

Discussion. The mechanism of attachment or binding of antibody molecules to cells (or of any body protein to cells) is unknown, though it is likely that specific binding and not simple adsorption of antibody upon the cell surface is necessary for sensitization to anaphylaxis(17,18). The inability of cortisone to interfere with the anaphylactic response of bowel muscle when started 18 hours after the antibody was given and the marked sensitivity of the uterine horns in the animals showing non-sensitized bowel strips suggest that cortisone may have interfered with the association of the antibody with the smooth muscle of the gut. The data do not suggest whether such interference is a specific action upon the binding of protein molecules to the cell or whether it is merely a manifestation of altered or depressed cellular function.

The amount of antibody N used for sensitization in most of these experiments, (25 γ), was chosen because this is the amount which will consistently passively sensitize 250 g guinea pigs to fatal anaphylaxis when given 48 hours before challenge. Although this quantity of sensitizing antibody is less than that used by others, it was chosen because cortisone does not exert all or none effects, and large excesses of antibody will mask cortisone actions.

We have no data to explain the paradox of the suppressive action of cortisone upon the smooth muscle of the gut with no demonstrable effect upon the smooth muscle of the uterus. (One can only speculate on whether the uterus, an organ which meets its greatest stress during labor, a time when large amounts of cortisone-like substances are circulating, has been spared an inhibitory metabolic effect of these hormones, while the bowel, an organ put physically at rest during stress, may also be metabolically depressed). The data do, however, suggest that cortisone may so alter some tissues that although adequate antibody is present for sensitization, no sensitization is elicited.

Summary. Administration of cortisone prior to and subsequent to sensitization of the virginal female guinea pig with known quantities of antibody, inhibited the response of the smooth muscle of the small bowel to antigen, but failed to alter the sensitization of uterine smooth muscle.

The author is indebted to Drs. Michael Heidelberger, Beatrice C. Seegal and Harry B. Van Dyke for their help in this work.

Received May 2, 1951. P.S.E.B.M., 1951, v77.

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19. Fischel, E. E., Stoerk, H. C., and Bjørneboe, M., in press.

Further Studies on Mechanism of Increased Capillary Permeability in Inflammation with the Aid of Cortisone and ACTH. (18859)

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It has been shown that adrenal cortical extract and cortisone suppress the increase in capillary permeability induced by leukotaxine or whole exudate(1,2). The increase in capillary permeability induced by commercial cortisone acetate is referable to the vehicle in which the cortisone is suspended and not to the material itself(3). More recently it has been demonstrated that whereas an alkaline exudate is inhibited from causing an increase in capillary permeability by cortisone(2), neither adrenal cortical extract nor cortisone have such an effect when the inflammatory reaction has attained an acid reaction. This is shown to be due to another factor released with the progress of an acute inflammation(4,5). This factor has been termed *exudin*(4). The combination of leukotaxine in the earlier stage of an inflammation and the liberation of exudin in the later phase of an acute inflammation offer a reasonable explanation for the mechanism of the increased capillary permeability throughout the duration of an acute inflammatory reaction. In early observations it was noted that the increase in capillary permeability induced by testicular extract was suppressed by adrenal cortical extract; however, the flare or spreading reaction was not inhibited(1). These observations have now been repeated with hyaluronidase. From 500 to 900 γ of hyaluronidase have been injected in conjunction with 2.5 mg of cortisone acetate. The injection of hyaluronidase was performed before, after or simultaneously with the cortisone acetate. These studies were made on several rabbits. It was found, for instance, that after injection of cortisone and hyaluronidase mixed together into the skin of the abdomen of rabbits, the subsequent intravascular injection of 5 cc of 1% trypan blue in saline is followed by no reduction in the spreading reaction. There seems at times to be a slight diminution in the intensity of increased capillary permeability as judged by the local accumulation of dye. The increase in capillary permeability often, but not always, follows the injection of such doses of hyaluronidase in the skin of the

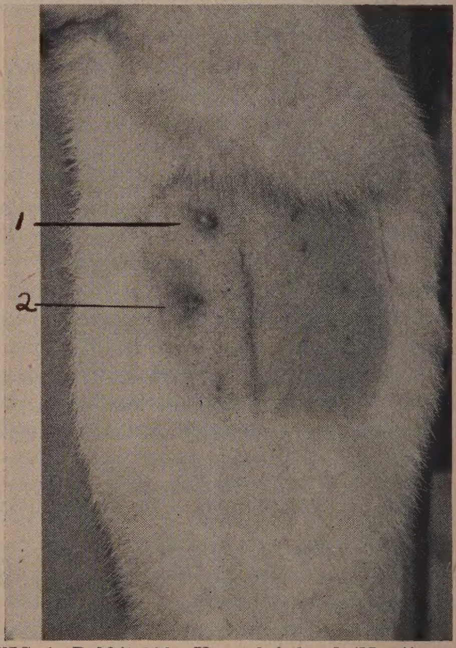

FIG. 1. Rabbit 302—Upper left hand (No. 1) represents effect on capillary permeability following injection of 2.5 mg of commercial cortisone acetate. Photograph was taken 1 hr after intrav. inj. of 5 cc of 1% trypan blue in saline and 1 hr and 13 min after the local inj. of cortisone. Lower left hand area (No. 2) represents combination of 2.5 mg of cortisone and 900 γ of hyaluronidase. Hyaluronidase was injected locally 2 min prior to injection of cortisone. Cortisone failed to abolish spreading reaction caused by hyaluronidase.

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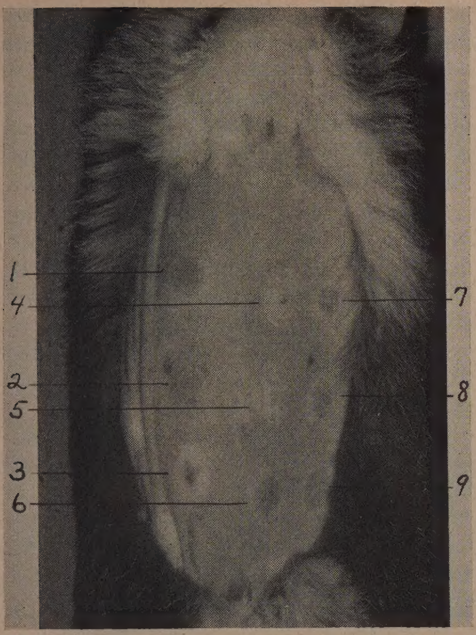

FIG. 2. Rabbit 296—Three rows of skin areas are shown as follows: *Left row,* reading up and down: Area 1—0.5 cc alkaline exudate from dog's pleural cavity. Sample of exudate is at pH 7.6. Note increase in capillary permeability to trypan blue as indicated by the local accumulation of dye. Area 2—0.5 cc of above exudate but converted to about pH 6.0 by addition of 10% lactic acid to sample. Although there is definite degree of necrosis by addition of lactic acid, the local accumulation of dye has occurred. Area 3—0.5 cc of adrenal cortical extract (Upjohn). Accumulation of dye has occurred only in center. *Middle row,* reading up and down: Area 4—0.5 cc from mixture of 0.5 cc of adrenal cortical extract plus 0.5 cc of alkaline exudate as used in Area 1. Note complete suppression of local increased capillary permeability as indicated by absence of dye. Area 5—0.5 cc from mixture of 0.5 cc of adrenal cortical extract plus 0.5 cc of converted exudate to about pH 6.0 as used in Area 2. In spite of acidity of exudative sample, the adrenal cortical material has to a large extent suppressed local accumulation of trypan blue from the circulation. Area 6—0.5 cc from mixture of canine exudate at pH 6.5 plus 0.5 cc saline. Local accumulation of dye, presumably referable to exudin present in acid sample of exudate. *Right row,* reading up and down: Area 7—Same as Area 6, but used only 0.3 cc of mixture. Effect is the same as in 6. Area 8—0.5 cc from mixture of acid exudate used in Areas 6 and 7 plus 0.5 cc of adrenal cortical extract. There is no suppressing effect to local increase in capillary permeability, referable to contained exudin in acid sample of exudate. Contrast this with suppressing effect of adrenal cortical extract utilized in Area 4 with an alkaline exudate, containing presumbly leukotaxine. Area 9—0.5 cc from mixture of acid exudate used in Areas 6, 7, and 8, plus about 700 γ of ACTH in 0.5 cc saline. There is some degree of suppression (*cf.* areas 6 and 9). Whatever dye accumulates in area may be due to some leukotaxine present in acid exudate and against the action of which, as far as only capillary permeability is concerned, ACTH seems impotent.

abdomen(6). The failure by cortisone to reduce the spreading reaction is illustrated in Fig. 1 (Rabbit 302). The details concerning the identification of exudin in inflammatory exudates which have attained an acid level have been described elsewhere(4,5). Exudin in contrast to leukotaxine is not suppressed by cortisone or adrenal cortical extract. This factor seems to be liberated in the final stage of the acute inflammatory reaction and its presence helps to explain the maintenance of capillary seepage during the period of the acute inflammatory reaction.

The acidity of the exudative material seems to contribute little to the effectiveness of exudin. An alkaline exudate can be converted from pH 7.6 to a pH of about 6.0 by the addition of 10% lactic acid, but nevertheless adrenal cortical extract will behave as if one deals with an alkaline exudate, *i.e.* by suppressing its capacity to induce increased capillary permeability. When exudin is present, the extract of the adrenal cortex is incapable of repressing the increase in capillary permeability. This is well illustrated in Fig. 2 (Rabbit 296). Conversely when an acid exudate, containing exudin, is rendered alkaline by the addition of .01 N NaOH, the resulting exudative sample is not repressed by adrenal cortical extract. It therefore seems as if exudin is primarily liberated at a stage which happens to coincide with the developing acidity in the inflamed area. The presence of exudin, in conjunction with the initial production of leukotaxine, offers a reasonable explanation for the increased capillary permeability throughout the length of the inflammatory process. The methods of isolation of exudin have been described elsewhere and will not be reiterated here(4,5).

A few of its biological and chemical properties may, however, be listed. In the first

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6. Menkin, V., *Newer Concepts of Inflammation,* C. Thomas, Springfield, Ill., 1950.

place exudin, similarly to leukotaxine, is not to be identified with histamine. Exudin is non-diffusible in contrast to histamine. It fails to produce the same pattern of tissue staining as with histamine(5). Finally, exudin when extracted for histamine by the method of Barsoum and Gaddum(7) fails to contract the isolated segment of guinea pig intestine. Subsequent addition of 1 mg histamine phosphate is accompanied by a marked contraction of the strip of intestine. This indicates that the absence of any refractoriness to the subsequent administration of histamine rules out that exudin is histamine.

The total nitrogen of exudin has been determined by the method of Pregl(8). The results obtained on 7 samples, as measured by Dr. W. Kalnins, may be tabulated as follows:

	Nitrogen (%)
	10.080
	9.230
	10.876
	9.880
	10.974
	10.960
	10.730
Avg	10.39 % N

The amino nitrogen values of exudin before hydrolysis and after hydrolysis have been determined by the copper method of Albanese and Irby(9). The findings can be listed as follows:

	Amino nitrogen	
	Before hydrolysis, mg/cc	After hydrolysis, mg/cc
	.00336	.00746
	.00317	.00746
	.02260	.05690
	.01306	.02615
	.00376	.00933
	.00466	.00746
	.00460	.00746
	.00280	.00653
	.00420	.00933
Avg	.00691	.01534

In brief, the available chemical data do not preclude the possibility that in exudin one is dealing with a dipeptide with a possible prosthetic group attached to it. The Biuret test is positive. However, further chemical studies will, it is hoped, yield more information as to the more precise nature of exudin.

Thus far it has been found that only one substance inhibits the increased capillary permeability caused by exudin. This is ACTH. Not only does ACTH inhibit locally the increased capillary permeability induced by exudin, as described elsewhere(10), but ACTH, in saline intravenously injected immediately prior to the local injection of exudin, likewise represses the increased capillary permeability elicited by the cutaneous injection of exudin in rabbits. The doses of ACTH employed ranged from slightly over 1 mg to 40 mg. Death at times followed the very large doses of ACTH. The data on 9 experiments are assembled in Table I.

It is evident from the results that the preliminary intravenous injection of ACTH suppresses in rabbits, the increased capillary permeability to trypan blue. A clear suppressing effect is detected when 10 mg of ACTH in saline is injected intravenously. This seems to be the threshold dose. When 35-40 mg of ACTH were administered, the lethal effect which sometimes follows seems referable to some type of intoxication. This part of the study requires further study. For instance, the blood flow with such large doses of ACTH has not been measured. When an acid exudate (Table I) is used besides exudin, it is found that intravenous injections of ACTH likewise reduce the increased capillary permeability to trypan blue. Presumably an acid exudate contains exudin, and this may well account for the effect obtained. When an exudate with an alkaline reaction is utilized the inhibiting effect of intravenously injected ACTH is not marked as with an acid exudate (Table I). It is probable that the presence of leukotaxine in an alkaline exudate may explain in part the incomplete suppressing ac-

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8. Pregl, F., *Quantitative Organic Microanalysis*, P. Blakiston's Son and Co., Inc., Philadelphia, 1937.

9. Albanese, A. A. and Irby, V., *J. Biol. Chem.*, 1944, v153, 583.

10. Menkin, V., *Am. J. Physiol.*, September 1951, in press.

TABLE I. Suppressing Effect of ACTH on Local Increased Capillary Permeability Induced by Exudin When ACTH Is Injected Intravenously.

		Readings on accumulation within about ½ hr after intrav. inj. trypan blue in exudin-treated skin areas:	
Exp. No.	Dose of ACTH or saline inj. intrav.	Experimental rabbits	Control rabbits
Exp. 1	1.4 mg ACTH in .9 cc saline	2+[‡]	
Control	.9 cc saline		3+
Exp. 2[*]	20 mg ACTH in 2 cc saline	0 in center of area; + to 2+ at periphery	
Control	2 cc saline		4+ at periphery
Exp. 3	20 mg ACTH in 2 cc saline	2+ in one area; trace in another area	
Control	2 cc saline		4+
Exp. 4	20 mg ACTH in 2 cc saline	+ at periphery	
Control	2 cc saline		2+ to 3+
Exp. 5	40 mg ACTH in 4 cc saline	0	
Control	4 cc saline		4+
Exp. 6 (.5 cc canine exudate—pH 7.2)	20 mg ACTH in 2 cc saline	Trace at periphery + to 2+	
Control (.5 cc canine exudate—pH 7.4)	2 cc saline		3+ 3+
Exp. 7[†] (.5 cc canine exudate—pH 6)	35-40 mg ACTH in ± 4 cc saline	0 0	
Control (.5 cc canine exudate—pH 6)	4 cc saline		3+ 4+
Exp. 8[†] (±.5 cc canine exudate—pH 6)	36 mg ACTH in 3.6 cc saline	0 0	
Control (.5 cc canine exudate—pH 6)	3.6 cc saline		2+ 3+
Exp. 9 (.5 cc canine exudate—pH 6)	10 mg ACTH in 1 cc saline	+ to 2+ 2+	
Control (.5 cc canine exudate—pH 6)	1 cc saline		4+ 5+

[*] Exudin utilized is in aqueous medium; in other experiments exudin is diluted with physiological saline (1:1) to approach isotonicity.

[†] These rabbits succumbed about 1 hr or over after the administration of 35-40 mg of ACTH. It is probable that death was referable to the large dose of ACTH injected. In one of those rabbits which was thoroughly autopsied one adrenal could not be found, and the other adrenal appeared as a mass of incongruous material which appeared, in the gross, very hemorrhagic and succulent unlike normal adrenal tissue.

[‡] The number of plus signs (+) refers to the extent of trypan blue accumulation into the treated skin areas from the circulating blood.

tivity of intravenously injected ACTH. In other words, cortisone inhibits the increase in capillary permeability caused by leukotaxine, whereas ACTH represses the increase in capillary permeability caused by another component liberated in an inflamed area, namely exudin. The suppressing effect of ACTH on exudin injected intravenously is illustrated in Fig. 3 (Exp. 5, Table I).

The significance of intravenously injected ACTH towards our further comprehension of the favorable effect of this substance in

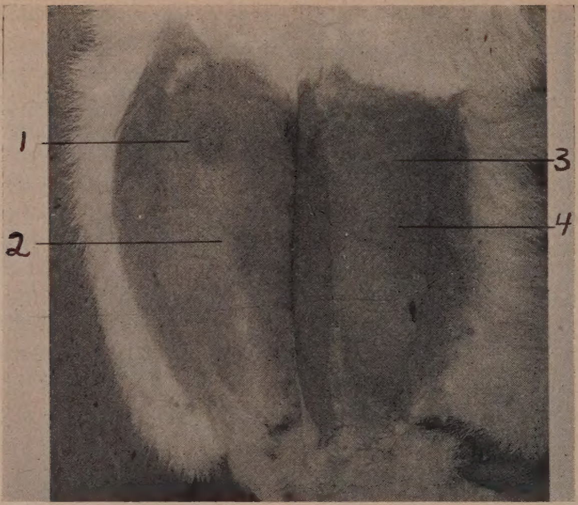

FIG. 3. Rabbits 1076 and 1077 (Exp. 5, Table I)—Two rabbits are placed side by side. *Right hand animal.* Has received 40 mg of ACTH intravenously in 4 cc of saline. 12 min later 0.5 cc of exudin was injected in areas No. 3 and 4 of skin of the abdomen. 6 min later about 5 cc of 1% trypan blue was injected intravenously. Even 25 min later there was no accumulation in exudin-treated skin area. Apparently systemic injection of ACTH suppressed the local accumulation of trypan blue injected intravenously. The animal was still alive 5 days later. *Animal on the left side.* Control rabbit (Rabbit 1076) was injected with 4 cc of saline intravenously. Two min later exudin was injected locally in skin areas No. 1 and 2 of the abdomen. 19 min later about 5 cc of 1% trypan blue in saline was injected intravenously. Two min later, there was local accumulation of the dye. This was very marked in 25-30 min. The photograph of both rabbits was taken about 2 hr later.

various infectious processes is suggestive. It is conceivable that by accumulating in a focus of inflammation from the blood stream, ACTH may reduce the effectiveness of the inflammatory process by mechanisms described in the present paper and elsewhere(11).

Summary and conclusions. Cortisone fails to obliterate the spreading reaction caused by hyaluronidase. This substance seems to inhibit at times somewhat the increased capillary permeability induced by 500 to 900 γ of hyaluronidase. An alkaline exudate or leukotaxine induces an increase in the local capillary permeability. Thus, as shown(1,2), the materials are inhibited by adrenal cortical extract or by cortisone. When, however, the exudate is acid in reaction, the local increased capillary permeability is not repressed by either cortisone or by adrenal cortical extract. This is shown to be referable to another factor liberated in the later stages of an acute inflammatory reaction. This substance is concerned with the mechanism of increased capillary permeability in inflammation. This factor is termed "exudin." Consequently the initial increase in capillary permeability in the development of inflammation is referable to leukotaxine. With the progress of the acute inflammation the pH of the exudate becomes acid and the mechanism of sustained increased capillary permeability is referable to exudin. The degree of acidity does not seem to be the cause of exudin formation. Exudin seems rather to be liberated about concomitantly with the development of an acid reaction in the inflamed area. The nitrogen content of exudin is about 10%. The amino nitrogen values before and after hydrolysis suggest that one is dealing possibly with a dipeptide to which there may be a prosthetic group attached. Further studies, however, will perhaps throw more light on this point. Exudin, tested for the presence of histamine, fails to contract

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11. Menkin, V., *Dynamics of Inflammation*, Macmillan Co., New York, 1940.

the strip of guinea pig intestine. When extracted for histamine by the method of Barsoum and Gaddum, there is no evidence that exudin contains any histamine. Its behavior toward cortisone and its indiffusibility indicate that leukotaxine and histamine are dissimilar in nature to exudin. Finally, the intravenous injection of ACTH suppresses the local increase in capillary permeability referable to exudin. The possibility of these findings in regard to a further understanding of the mechanism of ACTH in various infectious conditions are discussed.

Received May 28, 1951. P.S.E.B.M., 1951, v77.

Dermal Spreading of Hyaluronidase as Influenced by Prolonged Local Treatment with Certain Steroid Hormones.* (18860)

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Previous reports from this laboratory have demonstrated the increase in rate and alteration in pattern of dermal spreading by hyaluronidase as influenced by prolonged local (1) and parenteral(2) treatment with adrenocortical extract. As a result of these studies, it was desirable to examine the effects on enzymatic spreading of prolonged local pretreatment with some individual steroids.

Materials and methods. The steroids chosen for investigation were desoxycorticosterone,† 11-dehydro-17-hydroxycorticosterone (cortisone)† and progesterone.† The first 2 were supplied in the free alcohol form and were dissolved in 25% alcohol at a concentration of 1 mg/cc. The progesterone was dissolved in 50% alcohol at the same concentration. Twenty-one litter mate adult male rats of the Long-Evans strain in the weight range of 295 ± 75 g were used. For 5 months 0.1 cc of the desoxycorticosterone solution in 25% alcohol was applied daily to the dorsal cervical region just caudal to the right ear of 6 animals covering an area of approximately 10 sq cm; identical applications of the cortisone solution were made on 6 other animals. Four control animals were treated in a comparable site with 25% alcohol. Three animals were treated in the same manner and dosage with the progesterone preparation and their 2 controls received applications of 50% alcohol. In all 5 groups the left side of each animal was left untreated. Body weights were determined and regrown hair on the neck was clipped at weekly intervals after hair growth patterns had been recorded(3). The technic employed for study of the spreading action of hyaluronidase has been described in detail by Hayes and Reed(4). The hair was clipped closely from the proposed injection sites on each side of the neck and the animals were secured loosely for constancy of position. The animals were quieted by the subcutaneous injection of 0.3 cc of a veterinary solution of sodium pentobarbital (6.5% solution) and were anesthetized lightly with ether only for the duration of the initial injection. The indicator employed was an isotonic solution of hemoglobin. The final injection quantity was 0.05 cc containing 25 units of hyaluronidase.†

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* Supported by the Lawrence J. Montgomery Research Fund and a grant from G. D. Searle and Co.

1. Hayes, M. A., Reed, T. G., and Baker, B. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 361.

2. Hayes, M. A., and Baker, B. L., in press.

† The authors wish to express their gratitude to Dr. I. C. Winter, G. D. Searle Co., Chicago, Ill. for the hyaluronidase (Alidase), to Dr. J. M. Carlisle, Merck and Co., Inc., Rahway, N. J., for the 11-dehydro-17-hydroxycorticosterone, to Dr. Edw. Henderson, Schering Corp., Bloomfield, N. J., for the desoxycorticosterone and to Dr. F. E. Houghton, Ciba Pharmaceutical Products, Summit, N. J., for the progesterone.

3. Butcher, E. O., *Anat. Rec.*, 1934, v61, 5.

4. Hayes, M. A. and Reed, T. G., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 357.

The rate of injection was 0.01 cc/5 sec. The manipulation of the injection procedure was controlled carefully and intradermal injections were made in comparable sites on each side of the dorsal midline of the neck. One study was made on each side of each animal.

Measurements were made of the length and width of the ellipsoidal area of spread. The first measurement which was made at the termination of the injection was taken as a baseline. Then, observations were made every minute for 15 minutes; every 5 minutes through 30 minutes; every 10 minutes through one hour, and every 15 minutes through 2 hours. The increase in area for each time interval over that of the initial injection area was calculated. The arithmetic means were plotted, using the ordinate as the increase in area of spread over the initial area in sq mm and the abscissa as the logarithm of the time in seconds.

Results. There was no significant difference in the rate of body weight gain in any of the animals. Eighty-four per cent of the individual measurements in this entire study fall within the range of 4 sq mm of the plotted arithmetical means.

Complete inhibition of hair growth was produced by desoxycorticosterone and cortisone for 3 months prior to the period of testing. Progesterone, on the other hand, disturbed the symmetry of regrowth on the 2 sides but did not show evidence of inhibition. In the alcohol control animals no alteration in hair regrowth was detectable.

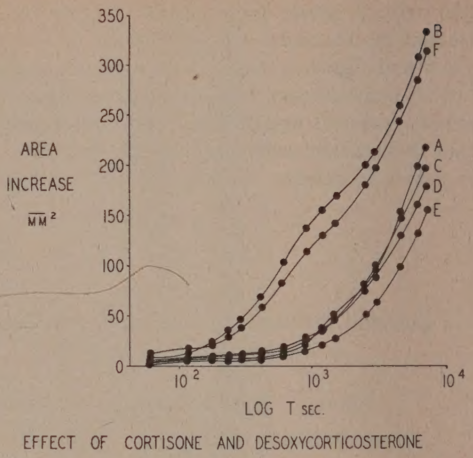

FIG. 1. Alterations in rate and pattern of spreading resulting from prolonged local pretreatment: Curve A, untreated side of desoxy animals; Curve B, treated side of desoxy animals; Curve C, untreated side of alcohol animals; Curve D, treated side of alcohol animals; Curve E, untreated side of cortisone animals; Curve F, treated side of cortisone animals.

Fig. 1 graphically presents the results of the hyaluronidase spreading activity as influenced by prolonged prior treatment with desoxycorticosterone and with cortisone. Curve B represents spreading action on the treated side of the desoxycorticosterone animals and curve F the treated side of the cortisone animals; curve A and curve E are their respective controls showing rate of spreading on the untreated sides of the same animals. Curves C and D illustrate the enzyme activity on the two sides of the alcohol control animals, respectively. It is apparent that there is no significant difference between right and left sides of alcohol control animals and the untreated sides of the experimental animals. The chief difference appears to be restricted to the area of steroid treatment resulting in a marked accentuation of enzyme activity and a significant alteration in the pattern of spreading as is apparent in the slow phase of spreading, as defined in other investigations reported from this laboratory(1,2,4).

In Fig. 2, on the other hand, progesterone pretreatment shows a different effect. In the slow phase of enzymatic spreading (to 10^3 sec) there is no significant difference between the treated and untreated sides of the experimental animals and either side of the alcohol control animals. The principal effect of progesterone has been a marked increase in enzymatic spreading in the rapid phase of spreading. The effect on enzymatic spreading was not influenced by the state of hair growth activity.

Discussion. Hayes and Baker(2) have suggested that adrenocortical hormones may affect the hyaluronidase enzyme-substrate complex in 2 antagonistic ways as measured by the dermal spreading of an indicator. Prolonged pretreatment locally(1) or parenterally(2) accentuates enzymatic spreading by altera-

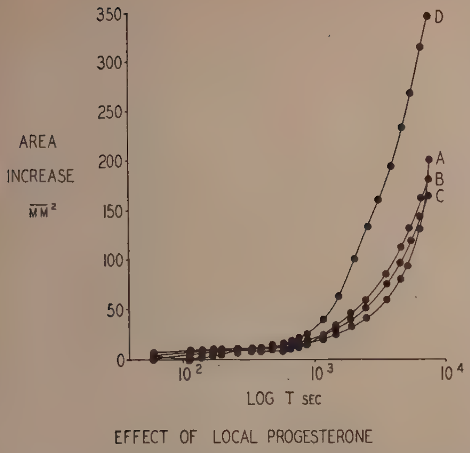

FIG. 2. Alterations in rate of spreading resulting from prolonged pretreatment: Curve A, untreated side of alcohol animals; Curve B, treated side of alcohol animals; Curve C, untreated side of progesterone animals; Curve D, treated side of progesterone animals.

tions in the connective tissue ground substance; acutely elevated levels of circulating adrenocortical hormones restrict enzymatic spreading by direct inhibition of the enzyme (2). Since these results may explain the contradiction concerning inhibition of enzyme activity by adrenocortical extract(5-7), it may also explain the contrary reports of inhibition by cortisone(5,6,8,9,10). The results of the present study demonstrate a marked *accentuation* in area of hyaluronidase spreading with all 3 steroids, desoxycorticosterone, cortisone and progesterone, as determined by measurements at 10^4 seconds. There is one great dissimilarity between the action of the steroids with an adrenal type of structure and the sex hormone; with the adrenal steroids, the slow phase of spread is modified so that it nearly has been abolished; while, with progesterone there is no difference in the slow phase of spread from any of the control situations.

It is interesting that prolonged pretreatment with desoxycorticosterone, the "salt active" steroid, or 11-dehydro-17-hydroxycorticosterone, the "carbohydrate active" steroid has an identical effect on hyaluronidase spreading of an indicator substance, particularly since Seifter, Baeder, and Begany(7) in an acute experiment, found synovial permeability to be increased to a maximum by either the enzyme or desoxycorticosterone. The individual effects were not additive with simultaneous administration of both enzyme and steroid.

The alteration in the slow phase of spreading as produced by the adrenal steroids and not by progesterone further strengthens the suggestion made by Hayes, Reed, and Baker (1) that the characteristic effect of prolonged treatment with adrenocortical steroids is on the slow phase of spreading activity.

Summary. An accentuation of hyaluronidase activity has been observed in skin which had been treated locally for 5 months with desoxycorticosterone, 11-dehydro-17-hydroxycorticosterone and progesterone. Only the adrenocortical steroids produced a characteristic alteration in the slow phase of spread. These alterations are interpreted as representing possible alterations in connective tissue ground substance, specifically related to steroids of adrenocortical origin.

Received May 28, 1951. P.S.E.B.M., 1951, v77.

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Some Factors Influencing the Total Phosphorus Content of Rat Uteri.
(18861)

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In the course of studies of the uptake of P^{32} by uteri of the albino rat(1), it was observed that a straight line relationship existed in certain instances between total phosphorus and wet weights of the uteri. There were no data available in the literature on this relationship. Studies were therefore outlined to extend these observations under varying conditions. These were to include the effect of estradiol, of hypophysectomy and of age on the phosphorus content of the rat's uterus as it related to the weight of the organ.

Materials and methods. Female albino rats of the Sherman and Wistar strain were used. Their diet was unrestricted and consisted of dog chow pellets, occasional bread and lettuce, and water. The animals comprised three groups: (1) immature (21-26 days old); (2) adolescent (approximately 2 months old); and (3) adult (3 to 7 months old). Hypophysectomy was done by the parapharyngeal route. If completeness of hypophysectomy was doubtful in any instance, the animal was not included in the study. Estradiol was injected subcutaneously in sesame oil or in dilute (20%) alcoholic solution. Two or 3 daily injections were given with doses ranging from 0.11 γ to 1.0 γ. Twenty-four hours after the last injection the animals were killed with chloroform, and the uteri were quickly excised and weighed. In some instances the uteri were expressed between filter paper. The uteri were each digested in 50 ml beakers with 5 ml of concentrated nitric acid on a hot plate at low heat. When almost dry, they were allowed to cool and 1½-2 ml of concentrated hydrochloric acid were added. After intermittent gentle heating until gas evolution ceased, they were taken almost to dryness, when 1½-2 ml water were added and the volume was again reduced to a few tenths of a milliliter. The phosphorus was then determined on a suitable aliquot by the method of Fiske and Subbarow (2), using an Evelyn Photoelectric Colorimeter with 660 filter. In taking up the contents of the beakers for analysis a small amount of carbon tetrachloride was added to

TABLE I. Data Regarding Groups of Animals and Manner of Treatment.

No. of rats	Age in days	Pituitary	Days after hypophysectomy	Uterus expressed	Treatment
10	21–26	Intact		No	None
8				”	Estradiol in oil .11–.21 γ daily for 2 days
5				Yes	None
3				”	Estradiol in oil .2 γ daily for 3 days
4	60–80			No	None
4	94–207			”	”
15				Yes	”
10				”	1 γ estradiol in oil daily for 3 days
8	21–26	Removed	7	”	Estradiol in oil .3–.4 γ daily for 3 days
4			7	”	None
2	94–207		39	No	”
8			6–55	Yes	”
8			24–146	”	Estradiol in oil 1 γ daily for 3 days
8			8–59	”	Estradiol in 20% alcohol .25–.5 γ daily for 3 days

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2. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, v66, 375.

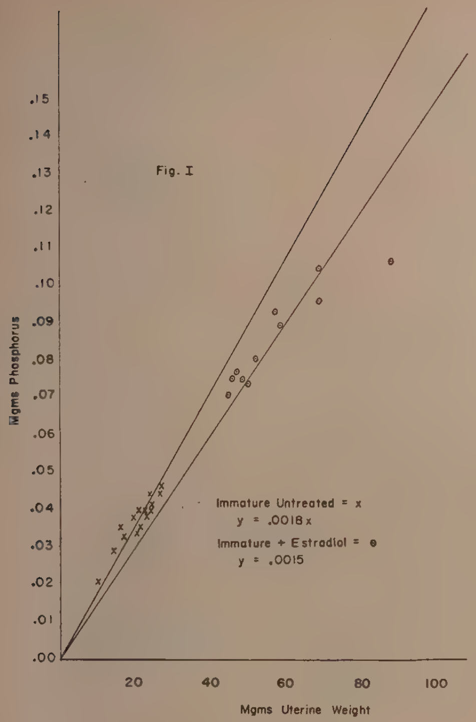

FIG. 1.

prevent occlusion by any residual fatty acid material. Since plots of the 8 sets of experimental data indicated the relationship to be of the general linear form $y = b + mx$, it was decided to evaluate the constants of the equation pertinent to each group of data. The boundary condition $b = o$ was applied; it was therefore necessary to compute "m" only. This was done using the method of least squares.

Results. Table I shows the numbers of rats, the ages in days, whether or not the pituitary had been removed and if so the time that elapsed, whether or not the uteri were expressed and finally, the treatment if any with estradiol.

The 4 figures show mg total phosphorus plotted against uterine weight in mg for the various groups. Fig. 1 shows the two curves $y = .0015x$ and $y = .0018x$ for the immature animals with and without estradiol respectively. There is a somewhat lower phosphorus content per mg of uterine weight in the estradiol treated animal than in the untreated animal. Fig. 2 shows the curves $y = .0024x$ and $y = .0015x$ for a small group of immature hypophysectomized animals without treatment and for a larger group treated with estradiol, respectively. Here the phosphorus content per unit weight of the uterus of the immature hypophysectomized untreated rats is significantly higher than those treated with estradiol. The curve for the estradiol treated hypophysectomized immature animals (Fig. 2) is identical in slope with that for the estradiol treated intact immature animals (Fig. 1). Fig. 3 shows the curves $y = .0016x$ and $y = .0017x$ for adult hypophysectomized rats with and without estradiol. The phosphorus content per mg of uterus is practically identical in these 2 groups. These slopes also are quite near the curve of the estradiol treated immature rats (Fig. 1). Fig. 4 shows the curve of the lowest slope, $y = .0011x$ with considerable scatter in the younger mature untreated intact animals. The equation for the adult intact estradiol treated animals is virtually identical, being $y = .0012x$.

Discussion. These data have been ex-

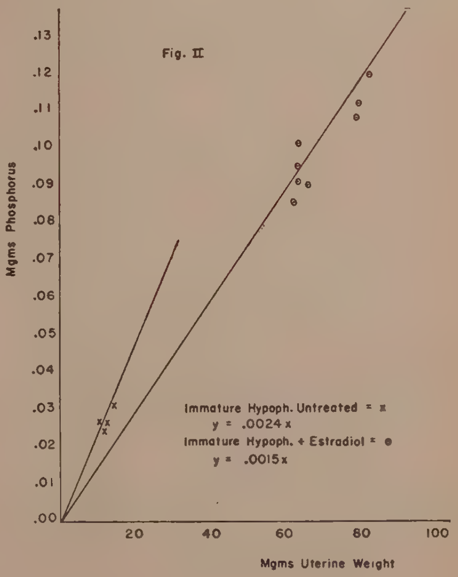

FIG. 2.

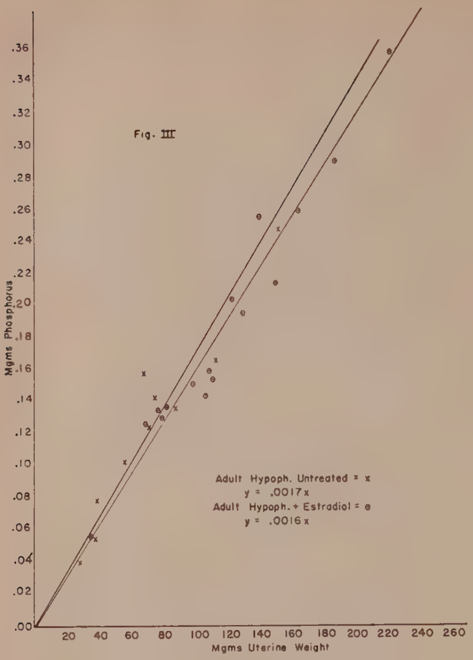

FIG. 3.

pressed in terms of simple straight line functions, y = mx, where m is milligrams of phosphorus per mg of wet weight of uterus. On this basis it was observed that the highest concentration of phosphorus occurred in the uteri of the immature hypophysectomized untreated rats. Conversely, the adult untreated rats and the adult estradiol treated rats showed the lowest phosphorus content per mg of uterine tissue (Fig. 4). Such relationships of phosphorus turnover to phosphorus content of the cells may be governed by hormonal factors under the influence of the pituitary gland. In this connection it may be pointed out that Gemzell and Samuels(3) observed that the rate of turnover of inorganic phosphorus in the rat was markedly reduced by hypophysectomy in all the tissues examined, but that it did not affect the concentration of the phosphorus.

The two factors to be considered in evaluating our observations are those of hypophysectomy and of the effect of estradiol on the phosphorus content of the uterus. Hypophysectomy causes reduction in metabolic processes in all of the tissues with a consequent retardation in turnover of the phosphorus in the uterus. The concentration of phosphorus in the immature untreated animal (Fig. 1) was highest after hypophysectomy (Fig. 2). This may represent gradations of the phosphorus content of the target organ (uterus) mediated through the stimulation of the ovaries by the pituitary. As the adult state of equilibrium is approached by the animals and the ovaries are producing endogenous estradiol, the phosphorus content per mg of uterine tissue decreases. The injection of exogenous estradiol in the adult rats did not appreciably alter this (y = .0011x and y = .0012x). Reference to Table II indicates these trends. The data were arranged in 2 groups; group A represents those animals that were untreated and group B includes those which received estradiol. In both groups age and/or hypophysectomy were the variables. The magnitude of the dose of estradiol was not an important factor.

The adult hypophysectomized animals and the immature intact animals had practically the same phosphorus concentrations in their uteri per unit weight of organ when they were treated with estradiol. Estradiol presumably causes an increased rate of phosphorus turn-

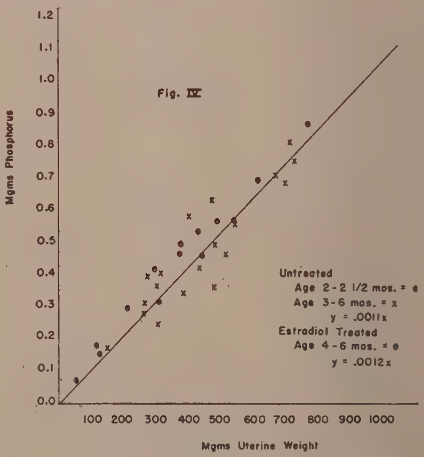

FIG. 4.

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3. Gemzell, Carl A., and Samuels, Leo T., *Endocrinology*, 1950, v47, 48.

TABLE II. Influence of Hypophysectomy and of Estradiol on Phosphorus Content of the Uterus.

Group A—Untreated	
Adult intact	$y = .0011x$
'' hypoph.	$y = .0017x$
Immature intact	$y = .0018x$
'' hypoph.	$y = .0024x$
Group B—Estradiol treated	
Adult intact + Est.	$y = .0012x$
'' hypoph. + Est.	$y = .0016x$
Immature hypoph. + Est.	$y = .0015x$
'' intact + Est.	$y = .0015x$

over in the uterus and overcomes the retarding influence of hypophysectomy on this target organ. It acts as an "equalizer" with respect to the phosphorus in the uterus of the immature intact, immature hypophysectomized, and adult hypophysectomized rat.

Investigations conducted in this laboratory showed the total phosphorus in the liver in both the immature and adult rat(4) to be the same per unit weight of tissue. The reason that the uterus does not act in this manner in the immature untreated rat as compared to the uterus of the adult rat may be explained by the fact that the uterus is a target organ, subject to changing metabolic processes with age. As maturity is approached the ovarian hormones, such as estradiol, may stimulate the phosphorus turnover and bring about an altered phosphorus content in the adult rat uterus.

From these observations it would appear justifiable to postulate that ovarian hormones, produced by way of pituitary activity, are important factors responsible for gradations of the phosphorus content of the uterus in varying conditions of age and glandular activity.

Conclusions. 1. The phosphorus content reaches a maximum per mg of uterine tissue in the immature untreated hypophysectomized animal. 2. The phosphorus content per mg uterine tissue is lowest in the adult and adolescent group in both the untreated and estradiol treated rats. 3. The phosphorus content per mg uterine tissue of estradiol treated animals is less than in the untreated animals. 4. Estradiol increases uterine weight and presumably stimulates phosphorus turnover but it does not increase the phosphorus content of the uterus. 5. The importance of estrogenic and pituitary hormones with respect to phosphorus metabolism in the uterus of the rat has been emphasized.

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4. Strickler, Herbert S., Cutuly, Eugene, Grauer, Robert C., and Wolken, Jerome J.; Unpublished data.

Received May 31, 1951. P.S.E.B.M., 1951, v77.

Evidence of Hypothalamic Control of the Pituitary Release of Thyrotrophin.* (18862)

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Although it has been postulated for many years that the hypothalamus in some manner regulates the secretion of thyrotrophin by the hypophysis, clear-cut experimental evidence demonstrating this phenomenon has not yet been presented(1). The present investigation was designed to provide a maximal stimulus for thyrotrophin release by feeding animals with hypothalamic lesions a diet containing propylthiouracil. In addition to thyroid size and histologic evidence of activity the iodide-concentrating capacity of the thyroid glands was determined, since this had previously been shown to be the most sensitive index of endo-

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* This work was aided in part by grants to Dr. E. B. Astwood from the American Cyanamid Co. and the U. S. Public Health Service.

† U. S. Public Health Service Postdoctoral Research Fellow of the National Cancer Institute.

1. Harris, G. W., *Physiol. Rev.*, 1950, v28, 139.

TABLE I. Data of Rats with Hypothalamic Lesions. T/S I^{131} ratio refers to the concentration of I^{131} in comparable quantities of thyroid tissue and serum.

Rat	Initial body wt	Final body wt	Thyroid wt, mg	Thyroid wt, mg/100 g body wt	T/S I^{131} ratio	Pituitary damage	Thyroid hyperplasia	Hypothalamic lesions
CR-1	251	277	14.3	5.2	22.3	Severe	Absent	Paraventricular to middle of ventromedian n. Extends through ventral surface hypothalamus bilaterally and more extensive rt. side.
CR-2	256	279	13.7	4.9	128	None	"	Suprachiasmatic to middle of ventromedian n. Extends across midline, more extensive on rt.
CR-3	239	256	14.1	5.5	247	"	"	Paraventricular to middle of ventromedian n.
CR-4	223	224	10.4	4.6	81	Small infarct left lobe	"	Middle to caudal end of ventromedian n.
CR-5	221	234	10.75	4.6	258	None	"	Beginning of paraventricular to ventromedian n. Extends across midline.
CR-6	258	249	16.4	6.6	215	"	"	Lesion very small, extending across midline at ventral surface of hypothalamus just behind suprachiasmatic n.
CR-7	255	223	19.1	8.6	433	"	Moderate	Paraventricular to ant. half ventromedian n. Lesions displaced to left.
CR-8	258	258	13.5	5.2	147	"	Absent	Paraventricular to anterior ventromedian n. Extends through surface of hypothalamus on left, 1 mm from surface on rt.
CR-9	246	252	20.15	8	395	"	"	Suprachiasmatic to anterior paraventricular n.

genous thyrotrophin activity available(2,3).

Materials and methods. Male and female Sprague-Dawley rats of the Harvard and Charles River strains were used. Hypothalamic lesions were produced in animals weighing 230-300 g by a modified Krieg stereotaxic instrument(4). Lesions were placed from 6 to 7 mm anterior to the ear plugs and 1 mm on each side of the midline. A varnished steel needle or copper wire with only the tip bare was used as the localizing electrode. After insertion through burr-holes in the dorsum of the skull, this was lowered until it encountered the base of the skull, when it was raised a distance of 0.5-1.0 mm. A direct current of 2.5-8.0 ma was applied for 15-30 seconds on each side. Ether was found to be a more satisfactory anesthetic than barbiturates.

After allowing one to 3 weeks for recovery from the operation to take place, the animals were given a diet of 0.03% propylthiouracil in mink chow and killed at the end of 10 days. The thyroids were dissected and weighed. The head was then removed and all excess tissue including the bony dorsum of the skull trimmed away, leaving the brain and base of the skull with the pituitary intact. All tissues were fixed in 10% formalin. After several days of fixing, 10 μ serial sections were made of the hypothalamic area, every tenth section

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3. VanderLaan, W. P., and Greer, M. A., *Endocrinology*, 1950, v47, 36.

4. Krieg, W. J. S., *Quart. Bull. Northwestern Univ. Med. School*, 1946, v20, 199.

TABLE II. Data of Control Rats.

		Thyroid wt,		
Rat	Final body wt	mg	mg/100 g body wt	T/S I^{131} ratio
1	296	41.4	14	456
2	290	34.15	11.8	298
3	278	29	10.4	232
4	286	44.1	15.4	283
5	307	50.9	16.6	242
6	260	28.2	10.9	285
7	315	35.3	11.2	261
8	296	34.75	11.7	270
9	262	25.2	9.6	283
10	275	35.8	13	343
11	251	31.2	12.4	239
12	271	44.55	16.4	290

being mounted. The pituitary and thyroid were sectioned separately, coronal pituitary sections at 10 μ being used in order to visualize both lateral lobes of the pars anterior adequately. The brain sections were stained with 1% toluidine blue for 6 hours while pituitary and thyroid were stained with hematoxylin and eosin. When determining the iodide-concentrating ability of the thyroid, one lobe was used for determination of radioactivity, the other being fixed in formalin.

The iodide-concentrating capacity of the thyroid was determined as previously described(3). Ten mg of propylthiouracil were injected subcutaneously 30-60 minutes before the injection of a 5 μc tracer dose of I^{131}. One to 2 hours later the animals were killed and the concentration of I^{131} per unit of thyroid tissues compared to the concentration in an equivalent amount of serum. This I^{131} was present only as inorganic iodide since the previous injection of propylthiouracil had effectively blocked all protein binding of iodine.

Results. Preliminary experiments in 19 rats had indicated that lesions in the rostral and tuberal mid-hypothalamic areas were most likely to give positive results. Accordingly, an attempt was made to place the lesions within the area bounded by the optic stalk and the infundibulum. Eleven animals were operated upon, of which 2 died a few days postoperatively. Twelve control animals of approximately the same weight were treated similarly except for the production of hypothalamic lesions. The results are summarized in Tables I and II.

It can be seen that a marked difference existed in the thyroid response to thiouracil feeding between the intact and operated animals. Whereas the thyroid weights of the control animals varied from 9.6 to 16.6 mg per 100 g body weight, averaging 12.8 mg, those of the hypothalamic animals ranged from 4.6 to 8.6, averaging 5.9 mg. The thyroids of the operated animals thus apparently did not undergo any appreciable hypertrophy with thiouracil administration, averaging less than half the size of the controls. In fact, they would seem to be even slightly smaller than the thyroids of untreated, intact rats in this laboratory, which usually weigh 7-8 mg per 100 g body weight.

Microscopic examination of the thyroid tissue revealed an atrophic, flat to low cuboidal epithelium in all operated animals except CR-7. In this one instance there was a moderate hyperplasia, although not nearly as intense as in the unoperated animals. The thyroids of the control group, on the other hand, all showed the usual marked hyperplastic changes seen in thiouracil-treated animals.

Perhaps the most surprising finding of the investigation was the marked increase in the iodide-concentrating capacity of the thyroid tissue in the absence of any anatomic evidence of hyperfunction. The thyroid:serum iodide ratio was well above the normal value of 25:1 in all but one animal and in most instances was fully as high as in the control thiouracil-treated animals. The one exception, CR-1, had a large infarct affecting both lateral lobes

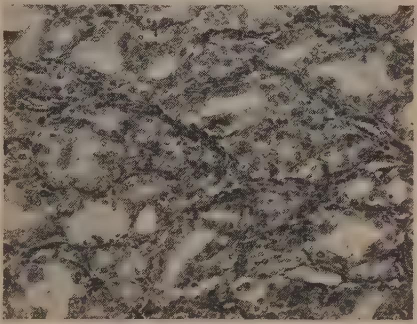

FIG. 1. Typical example of thyroid hyperplasia seen in control rats after 10 days of thiouracil feeding. Notice marked increase in cell height and diminution of colloid. H & E, × 160.

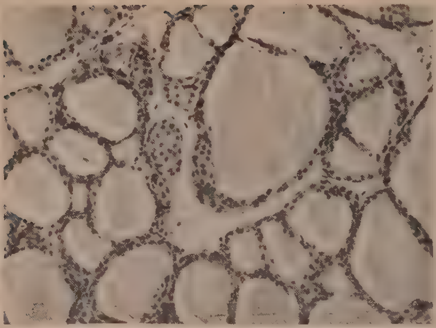

FIG. 2. Thyroid of CR-2. Atrophic appearance with large, colloid-filled follicles. Note flat, atrophic epithelium. H & E, × 160.

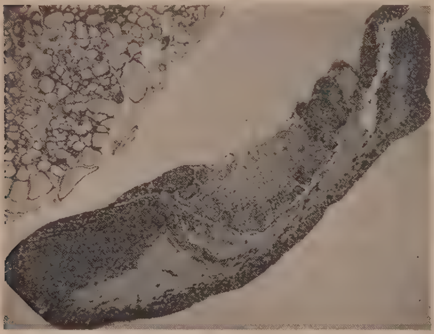

FIG. 3. Coronal section of pituitary of CR-1 showing large infarct affecting both lobes of pars anterior. H & E, × 26.

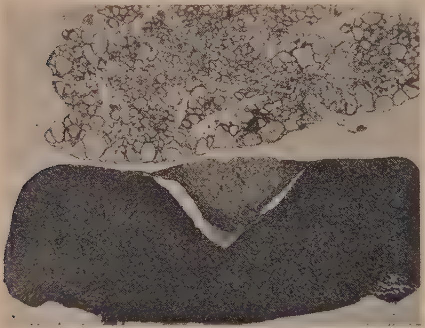

FIG. 4. Coronal section of pituitary and thyroid of CR-8 showing intact pituitary and atrophic thyroid. H & E, × 12.

of the anterior hypophysis, but nevertheless maintained a normal thyroid iodide concentration.

The pituitary glands appeared perfectly normal in all but 2 animals. CR-1 had a large infarct destroying most of both lateral lobes of the anterior hypophysis, as previously mentioned, and CR-4 had a small infarct in the left lobe of the pars anterior.

The hypothalamic lesions were of small to moderate size and were all located in approximately the area intended. The most anterior lesions were just behind the suprachiasmatic nucleus and the most posterior extended to the caudal portion of the ventromedian nucleus. The lesions in CR-1 with the large pituitary infarct extended through the ventral surface of the hypothalamus from the paraventricular to the middle of the ventromedian nuclei and that on the right side was more extensive. The largest lesions were in CR-2 and extended

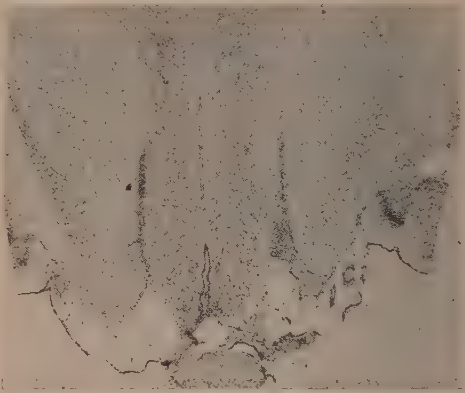

FIG. 5. Coronal section through hypothalamus of CR-4 illustrating bilateral lesions through caudal part of ventromedian nuclei. The infundibular stalk is in the lower midline. H & E, ×10.

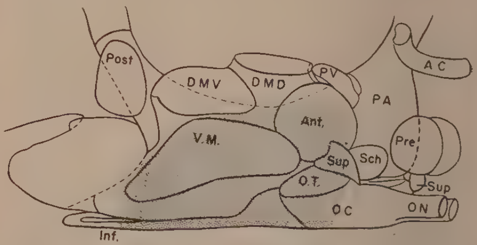

FIG. 6. Scheme of hypothalamus, modified from Krieg. Area of lesions indicated by stippling. A.C., anterior commissure; Ant., nucleus hypothalamicus anterior; D.M.V., D.M.D., ventral and dorsal portions of dorsomedian hypothalamic nucleus. Inf., infundibulum; O.C., optic chiasm; O.N., optic nerves; O.T., optic tract; P.A., nucleus hypothalamicus periventricularis, anterior region; P.V., paraventricular nucleus; Pre., preoptic nucleus; Post., posterior hypothalamic nucleus.

from the suprachiasmatic to the middle of the ventromedian nuclei. The lesions in this animal fused in the mid-line in the region of the anterior portion of the ventromedian nucleus, but did not extend through the ventral surface of the hypothalamus. The smallest lesion was in CR-6, extending across the ventral hypothalamic surface through the mid-line, just behind the suprachiasmatic nucleus. In CR-7, the only animal to show any evidence of hyperplasia of the thyroid epithelium, there was a displacement of the lesions to the left so that they were not bilaterally symmetrical. This was the only animal in which symmetrical lesions were not placed.

Discussion. It is evident that the animals with hypothalamic lesions did not show the usual goitrogenic response to propylthiouracil administration. Since the pituitaries of all but two animals were completely intact and only that of CR-1 showed severe damage, it is extremely unlikely that the absence of thyroid hypertrophy was due to pituitary injury. All animals had a final body weight equal to or greater than that at the time of operation. The one exception was CR-7, whose weight fell from 255 to 223 g. As described above, this was the only animal which showed moderate hyperplastic changes in the thyroid. The weight gain, although not marked, may in part have been due to lesions in the region of the ventromedian nucleus, since Brobeck(5) has established that damage to this area produces obesity.

It is difficult to explain the marked dichotomy between thyroid size and iodide-concentrating ability. An intact pituitary has been shown to be essential for both the goitrogenic response(6) and the increase in thyroid iodide concentration(3) following thiouracil administration. In this investigation it is evident that the usual increase in iodide concentration has occurred without concomitant anatomical evidence of hyperplasia. There has been some previous experimental evidence that these two features may be distinct in that the thyroid: serum iodide ratio falls much more rapidly following hypophysectomy in rats chronically treated with propylthiouracil than does thyroid size or cell height(3).

It seems reasonable to postulate that the hypothalamic lesions in some manner prevented the usual augmentation of thyrotrophin secretion in thiouracil-treated rats. Whether this was qualitative, quantitative, or both is not clear from the data available. It does seem evident that some pituitary thyrotrophin was being formed, since there was a marked increase in thyroid iodide concentration which would not occur in the absence of the hypophysis. Two possibilities might be suggested: (1) The pituitary is unable to secrete enough thyrotrophin to stimulate cellular hyperplasia of the thyroid but is able to secrete the smaller amount needed to increase the thyroid iodide concentration; (2) Two different thyrotrophins are secreted by the pituitary, one causing cellular hyperplasia and the other an increase in thyroid function. In the presence of hypothalamic lesions similar to those described above, the pituitary is able to secrete only the latter hormone. With the presently available evidence, it is impossible to state which, if either, of these theories is correct. Even the animal with severe pituitary damage was able to maintain a normal thyroid iodide concentration, so that in none of the animals was hypophyseal function completely absent. Normal animals fed a thiouracil diet for a comparable time immediately following hypophysectomy almost invariably have a thyroid:serum ratio below 10:1 and frequently closely approaching 1:1(7).

These experiments do not determine the nature of the hypothalamic influence on the pituitary. This may be either neural or humoral, but since most investigators have found only a very scanty nerve supply to the pars anterior(1) and since it is established that the hypophyseal portal system carries blood to the pituitary down the hypophyseal stalk(8), it would seem more probable that the influence might be humoral. Whether the hypothalamic area damaged exerts a primary influence on

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6. Astwood, E. B., Sullivan, J., Bissell, A., and Tyslowitz, R., *Endocrinology*, 1943, v32, 210.

7. Greer, M. A., *Endocrinology*, 1949, v45, 178.

8. Barnett, R. J., and Greep, R. O., *Science*, 1951, v113, 185.

the pituitary or only transmits tracts from other centers is also not clear.

The results of certain investigators with pituitary stalk-sectioned rats is compatible with the data obtained in this investigation. Brolin(9) found that the usual marked hypertrophy and hyperplasia of pituitary basophiles following thyroidectomy did not occur in stalk-sectioned rats and Greep and Barrnett observed a markedly decreased goitrogenic response following 2-3 weeks of thiouracil feeding in intact, stalk-sectioned rats(10). The latter authors, however, found that a marked infarction of the pituitary, similar to that seen in rat CR-1 of the present investigation, was produced by their technic of sectioning. Brolin did not state whether or not pituitary damage had been produced.

Summary. Bilateral, symmetrical, electrolytic lesions in the hypothalamus between the suprachiasmatic and caudal ventromedian nuclei prevented the goitrogenic response of the rat to thiouracil feeding. The iodide-concentrating capacity of the thyroid, however, increased to approximately the same extent as that of intact rats similarly treated. It appears that hypothalamic lesions of this type impair the secretion of thyrotrophin by the pituitary. It is not known whether this interference with thyrotrophin secretion is qualitative or quantitative, nor whether hypothalamic control is exerted by neural or hormonal mechanisms. The available evidence points to the secretion of a hypothalamic hormone, however.

I am indebted to Dr. E. B. Astwood for helpful advice and criticisms during the course of this investigation.

Received June 2, 1951. P.S.E.B.M., 1951, v77.

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Subcutaneous Administration of Combined Fat Emulsion with Hyaluronidase.* (18863)

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Hyaluronidase has been proved to be an important adjuvant for the rapid absorption of a wide variety of fluids injected subcutaneously. The principles and the technics for the introduction of fluids into the body by means of hyaluronidase have been applied in the present study to the administration of a combined fat emulsion developed in our laboratory. This emulsion has been previously reported upon with regard to its clinical effects when infused intravenously into human subjects(1,2).

Method of study. Hyaluronidase and the combined fat emulsion were infused subcutaneously into 22 patients. Each subject received one liter of the 10% combined fat emulsion. The latter contained 100 g of purified coconut oil, 50 g of the amino acids contained in commercial intravenous protein hydrolysate solution, 50 g of dextrose, and 30 g of gelatin as the stabilizing agent. It provided approximately 1200 calories per liter and the particle size of the emulsion averaged 1 μ. Hyaluronidase was injected immediately prior to the infusion in doses of 500 units into the anterior aspect of each thigh about 6 inches above the knee. The emulsion was infused into the subdermal tissue at rates varying from 4 ml to 7 ml per minute. All studies were begun with each patient in the basal or postabsorptive state and continued after the

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* Aided by a grant from the Abbott Research Laboratory.

1. Shafiroff, B. G. P., Mulholland, J. H., Roth, E., and Baron, H. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 343.

2. Shafiroff, B. G. P., and Mulholland, J. H., *Ann. Surg.*, 1951, v133, 145.

TABLE I. Summary of Data of Patient J.H. Who Received One Liter of the 10% Combined Fat Emulsion as a Clysis.

	Time in hr	Temperature, degrees F	Total lipid in blood serum, mg/%	Urine volume, ml	Total lipid in urine sample, mg
	–2	98.2	542	150	.6
	–1	98.8	475	—	—
Infusion period	0	99	350	72	7.7
	1	99	507	51	5.6
	2	99.2	623	22	2.6
	3	98.8	628	18	16
	4	98.6	725	16	3.2
	5	99	698	22	7.3
	6	98	634	26	.4
	7	100	—	31	1.6
	8	100	670	—	—
	9	100	—	—	—
	10	99.4	498	—	—
	11	100	—	94	.4
	12	100	530	—	—
	13	98.4	—	—	—
	14	99	460	103	6.4
	15	99	—	—	—
	16	99	—	—	—
	17	99	—	89	13.4
	18	98	464	—	—
	19	98.8	—	—	—
	20	98.8	—	90	3.5
	21	98.6	428	—	—
	22	98.6	442	—	—
	23	98.8	—	102	.7
	24	98.8	442	—	—

infusion through a period of 24 hours. Clinical records of temperature, pulse, blood pressure and respirations were taken at hourly intervals. Blood samples were taken on a regular schedule for the quantitative determination of total lipids(3). Urine samples were collected in fractional portions throughout the day and analyzed for their fat content(3). Quantitative estimations of oxygen uptake were made serially. Biopsy sections were taken through the infusion site for histo-chemical examination. For comparative purposes 8 of the patients in this series were also infused intravenously with the same type emulsion and identical studies as those above were made.

Results. The subcutaneous infusion of one liter of the 10% combined fat emulsion was well tolerated by all of the patients in this series. In 86% of the subjects there was a temperature elevation of one to 2 degrees above the initial pre-infusion temperature. In 6% the rise exceeded 2 degrees while in the other 8% the elevation was less than one degree. In all the subjects the temperature returned to its usual pattern at the end of the study period. There were no significant changes in blood pressure. Total blood lipids reached a peak level 30 minutes to 4 hours after the beginning of the clysis, varied in each case but usually attained the highest concentration early in the post-infusion phase of the study. The peak blood lipid value generally averaged twice that of the fasting level. At the end of the study period blood lipid values returned approximately to the initial levels in all cases. The rate of decline of total blood fat from the peak level varied in each case. The total amount of lipid recovered from the urine averaged 10 to 15 mg. One patient, a paraplegic, with severe kidney disease excreted a maximum of 69 mg of urinary lipids after the infusion of the emulsion. Oxygen consumption rose slowly during the course of the emulsion clysis, reached a peak level in the post-injection period and then tended to return to the initial level. The average maximum increase in oxygen uptake was 20%.

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TABLE II. Data from Same Patient J.H. Who Received Also One Liter of the 10% Combined Fat Emulsion Intravenously.

	Time in hr	Temperature, degrees F	Total lipid in blood serum, mg/%	Urine volume, ml	Total lipid in urine sample, mg
	−2	99	490	—	—
	−1	98.4	500	90	5
Infusion period	0	99	500	—	—
	1	100	570	—	—
	2	100	580	75	4.3
	3	100	678	50	6.6
	4	100	600	45	15.7
	5	100	—	—	—
	6	98.6	510	—	—
	7	100	515	60	5.1
	8	99.8	450	—	—
	9	99.2	408	170	3.5
	10	98.2	410	—	—
	11	98.8	—	200	2.4
	12	98.6	—	—	—
	13	98.4	420	—	—
	14	98.4	—	180	2.5
	15	98.8	—	—	—
	16	98.8	410	—	—
	17	98	440	75	.9
	18	98	520	—	—
	19	98.2	—	—	—
	20	98.2	500	98	.6
	21	98.4	—	—	—
	22	98.6	—	—	—
	23	98.6	—	—	—
	24	98.6	500	20	.8

No evidence of a toxic systemic reaction was noted nor was there any pathology at the site of injection that could be attributed to any of the constituents of the emulsion. The biopsy sections which were taken on the third post-infusion day were negative for tissue necrosis, granulomata or residua of fat. The latter observations were made from sections specially stained with Sudan III and oil-red-O. Included among the latter were biopsy sections from subjects who had received three consecutive clyses of the emulsion. Subjectively, the majority of the patients reported a sensation of warmth in the injected extremities and complained of tenderness to pressure over the infusion sites. Both of these symptoms subsided spontaneously within 2 or 3 days.

In the comparative tests with infusion by the intravenous route, temporary elevations of temperature developed similar to that previously described. Total blood lipids attained a higher peak level than that observed after hypodermoclysis and the curve of the rate of disappearance of fat from the blood seemed to follow a more constant pattern. Excretion of urinary lipids usually averaged less than 10 mg for the study period except for the paraplegic previously mentioned who again showed a greater loss of fat in the urine than the others. A progressive rise in oxygen uptake was found to occur more rapidly and the increments of increase were generally higher than those obtained after hypodermoclysis of the emulsion.

Discussion. This study has shown that the combined fat emulsion together with hyaluronidase can be successfully administered for nutritive purposes via the subcutaneous route. The facility for absorption of the combined fat emulsion can be accounted for by the action of hyaluronidase and its enzymatic hydrolysis of hyaluronic acid. Without hyaluronidase subcutaneous deposits of the emulsion were poorly tolerated in large amounts and were absorbed very slowly. Increase of total lipids in the blood, excretion of lipids in the urine and changes in oxygen uptake showed that absorption, diffusion and utilization of the combined fat emulsion were possible and effective through the subcutaneous pathway. The comparative studies indicated a parallelism with that obtained by the intravenous route.

The cause of temperature elevation and change in oxygen uptake in conjunction with clyses of the emulsion cannot be explained fully. The latter may be accounted for on the basis of a thermogenic response due to increased heat production associated with the combustion of fat. These changes may also be considered to be due to the presence of pyrogens in the emulsion. Should this be the case then the latter must be present in the coconut oil since the other ingredients have been proved to be pyrogen free prior to emulsification. Nevertheless the present combined fat emulsion is still being used clinically while these aspects of the problem undergo further investigation.

Summary. (1) A liter of a 10% combined fat emulsion was injected subcutaneously into hyaluronidase treated areas in each of 22 patients. (2) Each of 8 of the latter received the combined fat emulsion both by the intravenous and the subcutaneous routes. (3) Six patients in this series received 3 consecutive hypodermoclyses of the emulsion. (4) The general clinical reactions and the changes in blood lipids, urinary lipids and oxygen consumption were studied. (5) The clinical effectiveness of this type of administration for parenteral fat was established.

Grateful acknowledgment is made to Wyeth, Inc., for generous supplies of Aldase.

Received June 6, 1951. P.S.E.B.M., 1951, v77.

Effects of Enzyme Inhibitors on *in vitro* Dehydrogenase Activity of Cancer Tissue Slices.* (18864)

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During an investigation of the histochemical appearance of mouse tumor and control tissues incubated with TTC in the presence and absence of certain enzyme inhibitors it was found that these agents did not inhibit the reduction of 2,3,5-triphenyl tetrazolium chloride (*TTC*) by the tumor slices while inhibition of the control tissues was observed (1). It, therefore, appeared desirable to evaluate quantitatively the effects of several enzyme inhibitors on reduction of TTC by diverse human control and cancer tissues. Our findings indicate that under the experimental conditions employed fluoride causes less inhibition of the dehydrogenase activity of cancer tissue slices than it does in the homologous control material. Cancer tissue also shows minimal inhibition by malonate, but such findings may also occur in some normal tissues.

Materials and methods. Samples of human control and cancer tissues were obtained from surgical specimens within a half hour of the time of operation. The mouse tumors studied included spontaneous breast carcinomas arising in CFW and C3H strain mice as well as a transplantable mammary carcinoma grown in C57 black mice. Tissue slices, approximately 1 mm in thickness were prepared and placed in individual wide mouthed tubes containing the appropriate incubation mixtures, each of which contained 3 ml of a 1% aqueous solution of TTC buffered with phosphate buffers to pH 7.2. The different mixtures had in addition one ml of (a) 0.9% sodium chloride

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* Aided by grants from the Damon Runyon Memorial Fund for Cancer Research and the Leukemia Research Foundation.

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TABLE I. Reduction of TTC by Normal and Tumor Tissue With and Without Added Enzyme Inhibitors.

Tissue	Diagnosis	No. Cases	μg	TTC	Reduced/mg	
			R_O	R_F	R_M	R_{N_3}
Breast, human	Control	8	.8	.6	1.2	.4
’’ ’’	Carcinoma	28	.6	.6	.8	.5
’’ mouse	’’ *	19	1.3	1.3	1.5	1.4
Stomach	Mucosa	11	1.2	.7	1.1	1
’’	Carcinoma	5	.1	.1	.2	.2
Colon	Mucosa	11	1	.5	.9	.6
’’	Carcinoma	11	.7	.5	.7	.4
Kidney	Cortex	6	2.8	1.4	1.8	—
	Carcinoma	1	2.3	5.1	2.7	1.5
Lymph node	Control	1	.3	0	.7	—
	Acute leukemia	1	1.2	1.9	1.9	1.6
Thyroid	Control	15	.4	.3	.7	.3
	Carcinoma	2	.7	1.1	2.2	.9
Testicle	Control	1	4.5	4	4.5	4.5
	Embryonal carcinoma	1	1.1	1	1.2	.9
Metastatic ca.	Primary site unknown	3	1.1	1.3	1.3	1
Bone	Ewing’s tumor	1	.7	1.1	.7	.6
Skin	Melanoma	1	.5	.8	1	.3
Retroperitoneal granuloma	Non-specific	1	.6	0	0	—
Sacrum	Teratoma	1	1.1	1.1	2.4	1

* Data includes spontaneous breast cancer in C3H and CFW mice as well as a transplantable breast carcinoma.

(R_O), (b) 10^{-3} M sodium chloride (R_F), (c) 10^{-3} M neutralized malonic acid (R_M), and (d) 10^{-3} M sodium azide (R_{N_3}). The tubes were placed in a rack in an incubator, maintained at 37°C and subjected to continuous gentle agitation for a period of one hour. At the end of this time they were removed, the solutions decanted and subjected to acetone extraction of the reduced TTC. The concentration of reduced TTC in terms of μg per mg of acetone dried tissue was determined according to the technic previously described(2,3).

Results. In Table I we have indicated the extent of the TTC reduction by the various tissue slices incubated in the different experimental media. Where they were available we listed the homologous control and cancer tissue together. These data show that fluoride, in the concentrations employed, caused decidedly less inhibition of the cancer tissue than of the homologous controls (Fig. 1). In many instances actual increases in TTC reduction by cancer tissue slices were observed, particularly in the kidney carcinoma. Whereas the TTC reduction by inflammatory granulomas is markedly depressed or abolished, this is not true of fibrosarcoma tissue slices. This latter statement is based on histochemical observations of fibrosarcomas. Moreover, while carcinomatous tissue tends to show a slightly reduced endogenous dehydrogenase activity as compared to the control material, the reverse is true in sarcomas. Thus, normal lymph nodes and stromal tissue show only minimal reduction of TTC while lymphosarcoma and fibrosarcoma tissue are intensely stained. In common with carcinoma, such staining is not inhibited by fluoride under the experimental conditions employed.

There appears to be a linear relationship between the endogenous value and the amount of TTC reduced in the presence of fluoride (Fig. 2). While the relationship holds in the control and in the tumor tissues, the curves are separate and show increasing divergence with increase in the endogenous dehydrogenase activity. Although the individual values for the endogenous dehydrogenase activity within a particular tumor type may vary, the relationship to fluoride tends to remain constant. In Fig. 3 we have plotted

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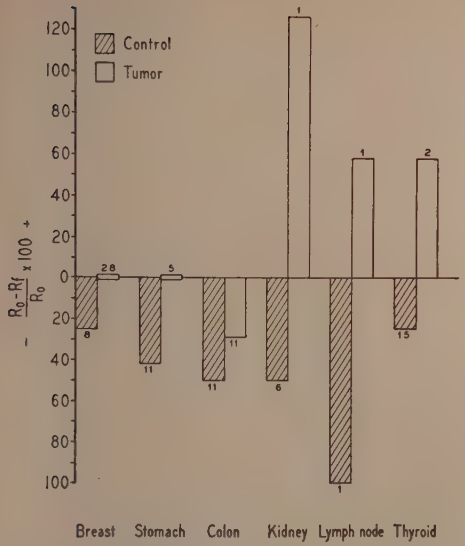

FIG. 1. % change in TTC reduction produced by NaF in homologous cancer and control tissue slices. Values in bars indicate No. of cases in each group.

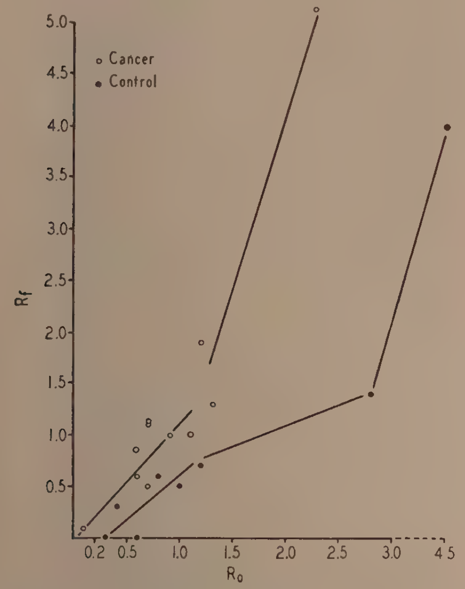

FIG. 2. Relationship between mean values for endogenous R_o and R_F reduction in control and cancer tissues.

such individual values for human and mouse mammary carcinoma; the same straight line relationship is obtained both for the human and mouse tissues.

Under the conditions employed, malonate failed to inhibit the various tumors and in many cases was associated with increased TTC reduction. However, some of the control tissues showed similar findings. In the experiments with sodium azide, the control and tumor tissues tested showed inhibition of varying degrees.

Special note should be taken of the findings in the mammary carcinoma of mice since these tumors tend to present a more uniform tumor cell population than obtains in human cancers. The absence of inhibition by fluoride, malonate or azide confirms our previous report based on frozen section examinations of the tissue slices(2). These findings were the same in spontaneous breast tumors arising in CFW and C3H mice as well as in a transplantable breast carcinoma grown in C57 black mice. This suggests that the data under discussion is not appreciably influenced by the host but reflects activity indigenous to tumor metabolism.

Discussion. The reduction of TTC by tissue slices provides a measurement of the dehydrogenase activity of viable tissues. While the particular substrates being metabolized during endogenous tissue respiration are not known, the effects of selected inhibitors of

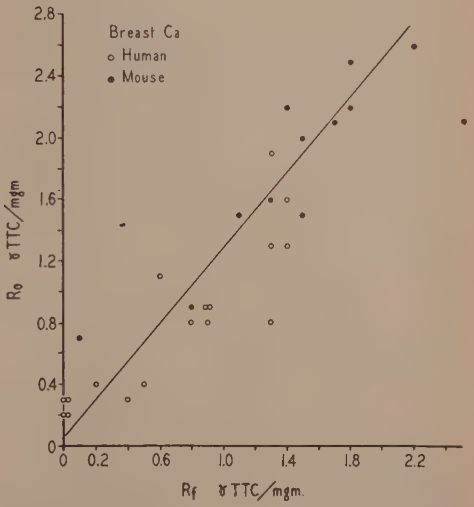

FIG. 3. Relationship between R_o and R_F values in individual cases of human and mouse mammary carcinoma.

enzyme activity on the TTC reduction may serve to characterize the type of metabolism in so far as such effects are reflected in oxidation-reduction reactions. Thus the experimental data presented may be considered in terms of pertinent information in the literature on biochemical features of cancer tissues. Among these are (a) tumors possess an increased aerobic glycolysis as compared to most normal tissues(4); (b) glycolysis in tumors appears to follow the Embden-Meyerhof schema(5-7); (c) tumors show minimal activity in regard to the citric acid cycle of reactions(8,9).

It has been shown that sodium fluoride may affect glycolytic reactions in two ways: (1) by inhibiting enolase and thus preventing the transformation of phosphoglyceric acid to pyruvic acid and (2) by inhibition of phosphatase activity. Since phosphatases may remove the phosphate from hexose diphosphate and other phosphorylated hexoses, they may limit the metabolic degradation of such compounds.

The enolase inhibition by fluoride would result in a decreased pyruvate formation. In so far as pyruvate is required to function as a H acceptor in the regeneration of oxidized coenzyme, previously reduced by the oxidation of phosphoglyceraldehyde to diphosphoglyceric acid, decreased available pyruvate would limit subsequent oxidation. Since it is mediated through a dehydrogenase such an effect should result in decreased TTC reduction. However, while most normal tissues remove pyruvate for participation in the reactions of the Krebs cycle, this occurs to a lesser degree in cancer tissue, thus minimizing the limited effect of enolase inhibition by fluoride. Moreover, the inhibiting effect of fluoride on dephosphorylations by phosphatases would provide more available substrate for triose formation and oxidation. From these considerations fluoride should result in an increased phosphoglyceric acid formation, and TTC reduction in cancer tissues. Novikoff *et al.* show in their Fig. 7 and 8 that fluoride produces such a change in homogenates of tumor tissue(5). Thus the increased TTC reduction found by us to occur in tumor tissue in the presence of fluoride is consistent with the Embden-Meyerhof schema of glycolysis and the known effects of fluoride on this mechanism. As judged by the general similarity of the curves shown in Fig. 2 a parallel relationship obtains in control and tumor tissues, suggesting that cancer tissue does not possess a different type of metabolism than is operable in most normal tissues.

It should be emphasized that we do not infer that the biological phenomenon of malignant neoplasia has been defined by quantitative determinations of the type reported or referred to here. The rapidly proliferating tissues of the normal testis and benign teratomatous proliferations may also show minimal fluoride inhibition under the conditions employed (Table I). While the data presented in the control and homologous tumor tissues appear to be related to the property of continued growth in the tumors, it cannot be said to reflect another fundamental property of cancer tissue, namely, metastatic potentialities. Biochemical representation of this phenomenon is still lacking and very much needed. This accords with our previous report on the features of morphology and metabolism in malignant neoplasia(10).

Summary. 1. Tissue slices from various human cancer and control tissues and mouse breast cancer were incubated in TTC solution with and without the enzyme inhibitors: fluoride, malonate and azide and the μg of TTC reduced/mg of acetone dried tissues determined. 2. Both control and tumor tissues exhibited a linear relationship between the endogenous and fluoride inhibited TTC reduction. The tumors were characterized by

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a decreased fluoride inhibition as compared to homologous controls. Malonate inhibition of tumor tissue also tended to be minimal. The findings were discussed in terms of the metabolism of tumor tissue.

Received June 7, 1951. P.S.E.B.M., 1951, v77.

Adrenal Cortex and Peptidase Activity of Rat Tissue.* (18865)

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There is general agreement that overactivity of the adrenal cortex results in a net increase in protein catabolism. This may result from a decreased rate of protein anabolism, an increased rate of protein catabolism, or both mechanisms may be operative(1). If the rate of protein breakdown were appreciably increased by adrenal cortical stimulation, a concomitant enhancement of proteolytic enzyme activity might not be unexpected. Therefore, an investigation of the effect of ACTH administration on the activity of several peptidases of various rat tissue homogenates and extracts was undertaken.

Materials and methods. Tissue extracts were prepared essentially as described by Smith(2). After a 48-hour fast, adult male rats of the Vanderbilt strain were anesthetized with nembutal, exsanguinated through the aorta, and one or more of various tissues were removed, rapidly weighed and transferred to a Waring blendor containing crushed ice and water. After mincing for 2 minutes, an aliquot of the homogenate was diluted with an equal amount of 0.02 M barbital buffer at pH 7.8 and refrigerated. The remaining homogenate was spun at 12,000 RPM for 30 minutes in a Servall centrifuge in a cold room at 4°C. An aliquot of the clear supernatant solution was similarly buffered at pH 7.8. Peptidase activity of these buffered mixtures was determined within a few hours after they were prepared. A modification of the procedures of Beloff and Peters(3) and Fruton(4) was used in preparing crude extracts of skin. About 5 g of skin was removed from the abdominal wall of an anesthetized rat. After subcutaneous tissue was scraped off, the skin was cut into strips about 2 mm wide. A coiled strip of skin was placed on a freezing microtome and the 50 μ sections taken were placed in 10 ml of 2% NaCl. The suspended sections were stirred for 3 hours at room temperature and then centrifuged at moderate speed for 30 minutes. Peptidase activity was determined on an aliquot of the clear supernatant solution. This entire procedure was carried out on skin obtained from 2 separate sites from each animal. The nitrogen content of unbuffered homogenate, supernate, and weighed portions of oven-dried whole tissue were determined by a semi-micro-Kjeldahl technic. Peptide hydrolysis proceeded in 10 ml Erlenmeyer flasks continuously shaken in a water bath maintained at 38°C. The hydrolysis mixture consisted of 0.5 ml of tissue preparation, 0.5 ml of 0.1 M substrate, 0.5 ml of 0.02 M barbital buffer (pH 7.8), and 0.5 ml of 0.85% N Cl. Hydrolysis mixtures containing glycyl-glycine as substrate were made 0.001 M with respect to cobalt. At 1, 2, and 18 hours, 0.2 ml samples were taken from the

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* This work was supported by grants from the National Institutes of Health of the Public Health Service, American Cancer Society, administered by Committee on Growth of National Research Council, and Duke University Research Council.

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TABLE I. Rat Tissue Extract Peptidase Activity.

Tissue	Treatment	LGGase				GGGase				GGase + Co⁺⁺			
		Homogenate		Supernate		Homogenate		Supernate		Homogenate		Supernate	
		C*	18 hr†	C	18 hr	C	18 hr	C	18 hr	C	18 hr	C	18 hr
Liver	N‡	2.3	27.5	3.6	25.1	.7	26.6	.8	24.2	1.4	24	1.7	25.1
		(5)	(5)	(5)	(5)	(3)	(3)	(3)	(2)	(3)	(2)	(3)	(2)
	ACTH	2.1	26	3	23.1	.5	22.8	.4	22.4	1.7	25.5	1.4	22.6
		(3)	(3)	(3)	(3)	(2)	(1)	(2)	(1)	(2)	(3)	(2)	(2)
Muscle	N	.3	24.3	.8	24	.1	24.6	.3	30.3	.2	24.5	.5	24.6
		(3)	(4)	(5)	(4)	(2)	(2)	(3)	(1)	(2)	(1)	(2)	(1)
	ACTH	.2	21.7	.8	23.7	.1		.3		.2	21.4	.4	24.1
		(1)	(1)	(3)	(3)	(1)		(2)		(1)	(1)	(3)	(2)
Lung	N	3	35.5	4	31.5	1.5	41.5	1.8	31.7	3.9	24.1	4.9	25.4
		(3)	(3)	(4)	(3)	(2)	(2)	(2)	(2)	(2)	(2)	(2)	(2)
	ACTH			3.1	34.6			.7	32.8			6.7	25.5
				(4)	(1)			(3)	(2)			(3)	(3)
Spleen	N	1.6	26	2.6	25.5	.7	28.6	.7	27.1	.9	25.3	1.1	25
		(2)	(2)	(3)	(2)	(2)	(2)	(4)	(2)	(2)	(2)	(2)	(2)
	ACTH			1.9	23.5			.8	21.5			1	23.5
				(2)	(2)			(2)	(2)			(2)	(2)
Kidney	N	2.3	34	3.1	35	1.8	42.3	1.5	34.8		25.5	6.3	23.7
		(2)	(2)	(2)	(2)	(2)	(2)	(4)	(2)		(1)	(3)	(3)
	ACTH			2.1	25.1			2.6	22			8.5	24.9
				(2)	(1)			(2)	(1)			(2)	(2)
Skin	N			.5	32							1.6	25.5
				(4)	(4)							(1)	(1)
	ACTH			.6	33.8							1.1	24.9
				(4)	(8)							(4)	(8)

* C = the number of micromoles of substrate hydrolyzed per min per mg of nitrogen contained in 1 ml of hydrolysis mixture, calculated on the assumption that cleavage of only 1 peptide bond occurs. Each value shown is an average of the number of animals shown in parentheses.

† 18 hr represents, for 1 ml of hydrolysis mixture the number of micromoles of substrate hydrolyzed in 18 hr assuming splitting of only 1 peptide bond. Each ml of hydrolysis mixture initially contained 25 M of substrate per ml. The numbers in parentheses represent the number of animals used for each average determination shown.

‡ N = normal.

hydrolysis mixture. The amount of substrate hydrolyzed was determined, in each sample, by a ninhydrin photometric procedure(5). From analysis of the 1 and 2 hour samples obtained and, assuming the cleavage of only 1 peptide bond, *the number of micromoles hydrolyzed per minute per mg of N contained in 1 ml of hydrolysis mixture* was calculated. This value, C, remained fairly constant over a 2 hour period, the first sample generally being somewhat higher than the second. In the interests of simplicity, only the average of the C values of the 1 and 2 hour samples is shown in Table I. A preparation of ACTH (Armour)[‡] was injected intraperitoneally 4-6 mg per day in 3 divided doses over a 7 day period. Rats so treated were fasted during the last 48 hours on ACTH administration and then sacrificed.

Results. As indicated in Table I, no striking changes in peptidase activity toward l-leucylglycylglycine (LGG), glycylglycylglycine (GGG) or cobalt-activated glycylglycine (GG) appeared in extracts or homogenates of tissues from animals receiving ACTH. Nor is there a definite trend discernible in the minor changes which occur. The range of values in control and ACTH-treated animals indicated that further investigation would not be fruitful. Moreover, in those instances where enough observations were made to make statistical analysis meaningful, P values ranged from >0.1 to >0.5. In addition, available data, which are not presented, clearly indicate that this lack of effect of ACTH administration on tissue extract peptidase activity re-

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5. Schwartz, T. B., and Engel, F. L., *J. Biol. Chem.*, 1950, v184, 197.

[‡] Kindly provided by Dr. John R. Mote of Armour Laboratories.

mains apparent irrespective of whether peptidase activity is calculated on the basis of nitrogen content of tissue, wet or dry weight of tissue or weight of the animals. It is of interest that, judging from the amount of hydrolysis occurring in 18 hours when the liberation of 25 μM/ml presumably indicates complete splitting of 1 peptide bond, lung and kidney extracts and homogenates were capable of hydrolyzing considerable quantities of dipeptide formed from the initial cleavage of LGG and GGG. This finding is supported by the fact that of all the tissues studied, *cobalt-activated* GGase activity is highest in lung and kidney.

Discussion. The hypothesis that the protein catabolic action of ACTH may be reflected, at least in part, in increased catheptic activity is not supported by this study. However, it may be that such changes in proteolytic enzyme activity, if they do exist, may be discernible only in viable tissue. This possibility is being explored using a procedure(6) which permits quantitative measurement of GGase activity in the surviving rat diaphragm.

Summary. The administration of ACTH to fasted rats has no appreciable effect on LGGase, GGGase and cobalt-activated GGase activity of crude extracts of liver, muscle, lung, spleen, kidney or skin.

The technical assistance of Mrs. Catherine Towbin and Mr. James F. O'Neill is gratefully acknowledged.

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Received June 8, 1951. P.S.E.B.M., 1951, v77.

Comparative Utilization of Carotene Administered Orally and Parenterally. (18866)

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The problem of utilization of carotene when administered parenterally to rats was shown by Tomarelli *et al.*(1) to be primarily a matter of the solvent used and the state of dispersion of the carotene. When carotene was solubilized in water by the use of a surface active agent (Tween 80), these workers found that one intramuscular injection of 0.5 mg carotene produced as good growth response in vit. A-deficient rats as did the same dosage given orally. In contrast, when carotene was dissolved in sesame oil and injected, the growth response and survival time were markedly reduced. We have confirmed these findings and have carried out experiments to determine the relative utilization of aqueous carotene when given orally and parenterally.

Methods. Male, weanling rats were caged individually and fed a purified vit. A-free diet composed of purified casein* 22%, salts(2) 4%, cottonseed oil (Wesson) 2%, vitamin-mix in casein 2%, and sucrose 70%. The vitamin-mix contained per 100 g: Thiamine hydrochloride 25 mg, riboflavin 50 mg, pyridoxine hydrochloride 25 mg, calcium pantothenate 250 mg, niacin 100 mg, folic acid 10 mg, biotin 10 mg, 2-methyl-1,4-naphthoquinone 25 mg, inositol 5 mg, p-aminobenzoic acid 50 mg, crystalline vit. B_{12} 0.005 mg, and choline chloride 5 g. In addition, 2 mg dl-α-tocopherol and 150 U.S.P. units of vit. D were added per 100 g ration. The food intake was restricted during the depletion period in an attempt to produce uniform weight gains. Purified, all-trans B-carotene(3) and crystalline vit. A acetate† were dissolved in water, using 20% Tween 40, or in cottonseed oil, by

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1. Tomarelli, R. M., Charney, J., and Barnhart, F. W., Proc. Soc. Exp. Biol. and Med., 1946, v63, 108.

* General Biochemicals, Inc.

2. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, v138, 459.

3. Bieri, J. G., *J. Nutrition*, 1951, v44, 327.

the procedure described previously(3). dl-α-tocopherol was added to the supplements so that the concentration was 0.3%. The preparations were stored under nitrogen at 7°C. After 19 days, when the animals had ceased to gain for three consecutive days, and many had developed xerophthalmia, they were divided into groups of similar average weights. The ration, from which the α-tocopherol was now removed (except for the control animals), were given *ad lib*. Supplements of carotene or vit. A in 0.1 ml oil or water (containing 0.3 mg α-tocopherol) were given daily for 28 days. Oral supplements were given by stomach tube using a dull, bent number 16 hypodermic needle. The intramuscular injections were given in the thigh muscle, alternating the legs daily. It was found that the oily preparations frequently leaked from the site of injection, especially after the first week. This was seldom encountered with the aqueous preparations except during the last several days of the experiment. To prevent the oral ingestion of any injected supplement which may have exuded, the animals were inspected within a few minutes after injection, and any leakage was carefully washed off.

Preliminary experiments revealed that over a 21-day period, 10 γ of carotene in Tween injected intramuscularly, daily, produced as good growth as did 1.8 γ orally (average of 4.1 and 3.9 g daily, respectively). A dosage of 5 γ intramuscularly gave a smaller response, 3.3 g daily. When dissolved in oil, 15 γ carotene injected daily failed to maintain weight. In the subsequent experiments, smaller supplements of aqueous carotene were used to determine the effectiveness of low dosages when given parenterally.

TABLE I. Comparative Utilization of Carotene in Water (20% Tween 40) or Cottonseed Oil by Vit. A-Deficient Rats; 28 days.

Group	Supplement	No. rats, No. survivors	Avg daily wt gain (survivors)
I	0	5/0	—
II	1.6 γ carotene in Tween, IM	9/6	2.2 ± .27*
III	4.2 γ carotene in Tween, IM	10/8	3.2 ± .22
IV	1.6 γ carotene in Tween, oral	9/6	4.1 ± .18
V	1.5 γ vit. A in Tween, oral	10/9	3.9 ± .33
VI	24.6 γ carotene in oil, IM	8/1	1.6

* Standard error $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

Table I shows the various supplements, their route of administration and the survival and growth of the rats for the 28-day period. The daily injection of 1.6 γ (group II) and of 4.2 γ (group III) of carotene solubilized in water produced growth responses of 2.2 g and 3.2 g daily, respectively. When 1.6 γ of aqueous carotene were given orally (IV), the average daily gain was 4.1 g. This is probably the maximum growth obtainable, as shown by the growth of rats in group V receiving 1.5 γ of aqueous vit. A orally. From a comparison of groups III and IV, it is apparent that 4.2 γ of carotene when injected is slightly less active than 1.6 γ orally. Inasmuch as the preliminary experiments showed that 10 γ of carotene injected daily produced maximum growth, while 5 γ did not, it would appear that from 4 to 6 times as much carotene is required parenterally as orally for a similar growth response.

Further evidence that carotene in oil is not utilizable when injected is shown by group VI, in which all but one rat died before the end of the experiment. At autopsy, these rats had large deposits of oily carotene at the site of injection, as has been reported by other observers(4,5). The death of some of the rats in those groups apparently receiving adequate carotene or vit. A is attributed to the fact that at the beginning of the experiment, some of the animals were in an advanced stage of vit. A-deficiency, and could not respond to the supplement. At the end of the experiment, the surviving animals were sacrificed

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† Kindly supplied by Hoffmann-La Roche, Inc., Nutley, N. J.

4. Lease, V. G., Lease, E. J., Steenbock, H., and Baumann, C. A., *J. Lab. and Clin. Med.*, 1942, v27, 502.

5. Sexton, E. D., Mehl, J. W., and Deuel, H. J., *J. Nutrition*, 1946, v31, 299.

TABLE II. Utilization of Carotene in Water (20% Tween 40) Administered Subcutaneously to Vit. A-Deficient Rats; 21 Days.

Group	Supplement	No. rats No. survivors	Avg days survival	Avg daily wt gain
I	0	5/1	11	—
II	.1 ml aqueous 20% Tween 40, oral	6/0	14	—
III	9.6 γ carotene in Tween, subcut.	5/5	—	4.7

and the livers and kidneys analyzed for vit. A and carotene. Small amounts of carotene (6-9 γ, total) were found in the livers of the group receiving 4.2 γ carotene by injection, and only traces of the pigment were detected in the group injected wth 1.6 γ carotene. Neither vit. A nor carotene was found in the kidneys of these two groups. The tissues of the other groups contained no detectable vit. A or carotene. Examination of the thigh muscles of the rats receiving injections of aqueous carotene revealed no apparent atrophy. The muscle appeared to be torn, however, presumably due to the repeated insertion of the needle. A light yellow coloration throughout the tissues of the leg indicated that the carotene was not completely absorbed.

A second experiment was performed to determine if aqueous carotene when injected subcutaneously would also be effective in curing vit. A-deficiency. Three groups of vit. A-deficient rats were placed on the vit. A-free ration described above. Group I, the controls, received vit. E in the food, while groups II and III received this vitamin in the supplement. Group II was given daily 0.1 ml of 20% Tween 40 in water, orally, while group III received 9.6 γ of carotene in 0.1 ml of water (20% Tween 40) subcutaneously. The results in Table II show that the Tween solution (group II) had no appreciable effect on the survival of vit. A-deficient rats, and that subcutaneous, aqueous carotene (group III) is well utilized.

Summary. Carotene solubilized in water by the use of Tween 40 will support growth in vit. A-deficient rats when as little as 1.6 γ is injected intramuscularly daily. For maximum growth, approximately 4 to 6 times as much carotene is required parenterally as orally. Aqueous carotene is also well utilized when given subcutaneously. Carotene dissolved in cottonseed oil and administered parenterally is essentially not utilizable.

Received June 12, 1951. P.S.E.B.M., 1951, v77.

Developmental Abnormalities in the Chick Embryo Following Infection with Newcastle Disease Virus. (18867)

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The report of Gregg(1) that maternal rubella may act as a teratogenic agent in human subjects, has focused interest on the possible role of virus infections in the production of congenital abnormalities. In efforts to pursue the study of teratogenicity of viruses, the following laboratory experiments were planned to observe the effects of viral agents on the developing chick embryo. Initial investigations using fowl pox virus were inconclusive. However, Newcastle disease virus produced a high percentage of developmental defects, particularly in the lens of the eye and in the auditory sac.

Methods and materials. The Newcastle disease virus was supplied by Dr. F. R. Beaudette.* Fertile eggs from New Hampshire

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1. Gregg, N. McA., Trans. Ophthalmological Soc. of Australia, 1941, v3, 35.

Red chickens were incubated in a forced-draft incubator at 99°F. Virus and control inoculations were made at 45 hours of incubation. By opening all of the eggs and preparing them with cover slips immediately prior to the time of inoculation, all virus and control injections in each experiment were completed within one hour. The virus inoculum consisted of allantoic fluid from a 12-day embryo inoculated 24 hours previously with Newcastle disease virus. Control inoculum consisted of allantoic fluid from a normal 12-day embryo. .01 ml of inoculum was injected beneath the vitelline membrane over the blastoderm near the edge of the area pellucida, the flow from the needle directed toward the embryo. In preliminary experiments virus inoculated embryos uniformly died within 24 to 36 hours. To make valid their study for developmental abnormalities, embryos were harvested alive 20 to 24 hours after inoculation. (This seemed important in light of observations(2) in chick embryos that postmortem changes may simulate developmental defects.) Embryos were fixed in Bouin's solution, stained in dilute alum cochineal, cleared and mounted *in toto* for examination under the dissecting microscope.

Although individual variation in developmental progress of chick embryos of the same incubation setting occurs, the majority of the embryos at the time of inoculation corresponded to developmental Stage 11 as described by Hamburger and Hamilton(3). At this stage the olfactory placode is absent, the auditory vesicle is a wide-open pit, the optic vesicle and optic stalk are present but invagination of the primary optic vesicle and the appearance of the lens placode begins a few hours later. The amnion does not yet cover any of the embryo. At the time of harvest, 20 to 24 hours later, the embryo corresponds approximately to developmental Stage 16 or 17. At these stages the amnion may extend to the 26th somite or may be completely fused over the embryo, the auditory vesicle is an almost closed sac, the double walled optic cup is established, and the mouth of its cavity is practically filled by the lens in the form of a thick walled epithelial sac. The olfactory placodes appear as thickenings of the ectoderm or as shallow pits in the thickened ectoderm.

Results. In the first 150 embryos infected with N.D.V. 42% showed abnormalities of the lens or auditory sac or both. Of the 150 control embryos inoculated with virus-free material, no similar defects were found. In certain subsequent experiments the specific abnormalities were obtained in 80% to 90% of the embryos following inoculation with N.D.V. Virus samples of such known teratogenicity, when stored in hermetically sealed ampules at —50°C, retained these teratogenic properties for at least four weeks. In 75 embryos infected with samples of stored virus 90% showed the defects. More extensive studies are in progress.

General retardation in growth of the embryo and varying degrees of retardation of the amnion were observed in some of the embryos but the outstanding abnormalities were seen in the embryonic lens and the auditory sac (otocyst) ranging from obvious reduction in the size of the two organs to their complete absence. Both defects were found to occur bilaterally or unilaterally. A control embryo showing the normal appearance of the structures concerned is shown in Fig. 1, and embryos showing typical abnormalities are shown in Fig. 2. In embryos in which the lens or

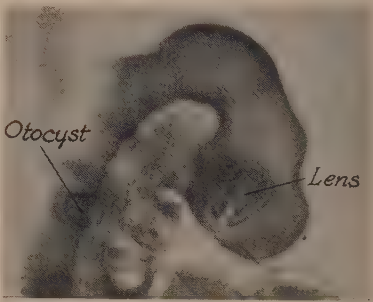

FIG. 1. Normal embryo of 65 hr incubation.

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* F. R. Beaudette, Poultry Pathologist, N. J. Agric. Exp. Station, Rutgers University, New Brunswick, N. J.

2. Blattner, R. J., and Williamson, A. P., *Arch. Path.*, 1950, v50, 676.

3. Hamburger, V., and Hamilton, H. L., *Jour. Morph.*, 1951, v88, 49.

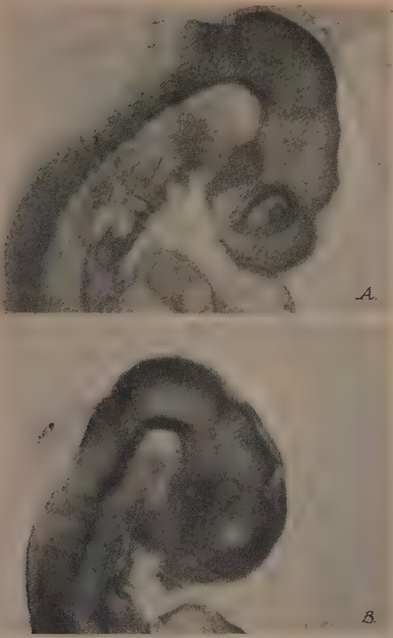

FIG. 2. Newcastle disease infected embryos of 65 hr incubation. Upper photograph demonstrates small lens and small otocyst. Lower photograph shows absence of both lens and otocyst.

otocyst was present but abnormal in structure the defect appeared to consist of significant retardation in morphological differentiation as well as reduction in size of the organ. Although in most instances the optic cup appeared normal, in one or two embryos it appeared slightly distorted. It must be emphasized that although general retardation of growth appeared in some of the embryos, the majority of those showing specific defects of the lens and otocyst were in the same stage of development as the control embryos with respect to visceral arches, limb bud differentiation, and somite count. A typical example of such observations is demonstrated in Fig. 3.

Microscopic examination of serial sections of 4 infected embryos confirmed the conclusions based on observations of the whole mounts. In all embryos studied the otocysts or lens, or both were missing, or severely damaged and markedly retarded. In two embryos the olfactory plates were absent. Detailed study of these microscopic findings is in progress. Preliminary studies of the lens defects suggest findings in autopsy studies of human fetuses from maternal rubella cases in which retardation of the lens was reported by Cordes and Barber(4) and necrosis and degenerative changes were described by Swan(5).

Summary. (1) The lens and auditory sac showed defective development or were completely missing in 42% of 150 chick embryos infected with Newcastle disease virus at 45 hours of incubation. In 150 control embryos, inoculated with normal allantoic fluid, no similar defects were found. (2) In subsequent experiments the specific defects were obtained in 90% of the N.D.V. infected embryos. Virus samples of such known teratogenicity if stored in hermetically sealed ampules at —50°C retained their teratogenic properties for at least 4 weeks.

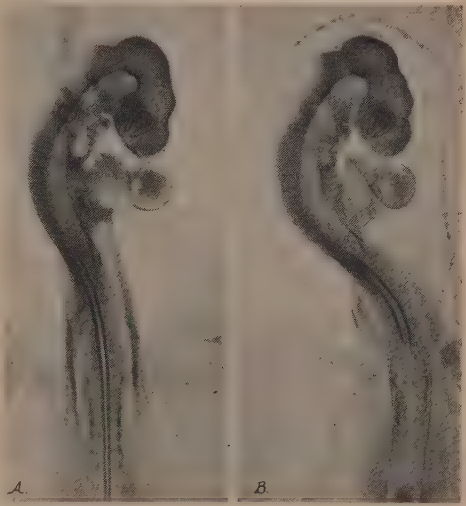

FIG. 3. Embryos of 65 hr incubation. A, normal; B, infected with Newcastle disease virus.

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Received June 13, 1951. P.S.E.B.M., 1951, v77.

A Tracer Study of Distribution of Iodoacetate in Rat Tissues.* (18868)

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In the course of a study of the mechanism by which iodoacetate inhibits dental caries, it was found desirable to determine the distribution in rat tissues of iodoacetate labelled with I^{131}. Since there is a possibility of slight decomposition of iodoacetic acid with liberation of iodine, it was necessary to make a comparative determination of the distribution of I^{131} as iodide in order to be sure that the observed results were due to iodoacetate rather than to iodide in the tissues. Previous studies of the distribution of I^{131} in rabbits(1,2) and rats (3) have indicated that, except in the thyroid gland, there is no selective uptake of radioactive iodine by body tissues.

Experimental. Synthesis of labelled iodoacetic acid. The method was based on that of Guggenheim(4). An aqueous solution of 200 mg of potassium iodide and 200 mg of monochloroacetic acid to which had been added 1.75 millicuries of carrier-free I^{131} was heated one hour at 50-60°. Sodium bisulfite was then added to remove any free iodine, and the solution was twice extracted with ether. The water layer was treated with 50 mg of iodoacetic acid and once more extracted with ether. The ether extracts were evaporated to dryness, and the residue taken up in distilled water and diluted to the desired strength. Yields averaged 48%. The freshly prepared solutions gave no color with starch, and were used immediately. When the solutions were allowed to stand for a week, enough iodine was liberated to give a faint starch test.

Distribution studies. Mann, Bale, Hodge, and Warren(2) showed that the presence of I^{127} reduces the uptake of I^{131} by the tissues. In order to compensate for the possibility of I^{127} which might have accompanied the iodoacetate through the procedure for its isolation, the iodide solutions to be used were prepared as follows: A solution of 400 mg of KI in 100 cc of water was extracted 3 times with ether. The ether was evaporated and the residue taken up in water to which the carrier-free I^{131} was added. Any I^{127} which was carried through the process should then be present in both the iodide and iodoacetate solutions in comparable amounts.

Toxic effects were noted in rats when iodoacetate was injected in concentrations slightly under 50 mg per kg. In preliminary studies to determine the time required for maximum uptake of iodoacetate by the teeth, 10 rats weighing approximately 100 g were injected intraperitoneally with about 4 mg of iodoacetate having an activity of 15-20 microcuries. Two rats were sacrificed at 1, 3, 6.5, 24, and 50 hours each. The tissues to be studied were removed, weighed fresh and dried at 56° for 48 hours. After determining the dry weight, they were ground to a fine powder and placed under a Geiger-Muller tube for counting in porcelain planchets having an area of 14 cm². Each sample was counted for 30 minutes. This allowed for a 5% error in counting statistics as all samples were from 4 to 18 times above background count. An aliquot of the dose given to the animals was run concurrently with the samples. This obviated any calculation that would otherwise be necessary in correcting for the decay of the I^{131} in the iodoacetate

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* This work was supported by grants from the Division of Research Grants and Fellowships of the National Institutes of Health, Bethesda, Md. and from the California State Dental Association. The I^{131} was supplied by the Oak Ridge National Laboratory on allocation from the Isotopes Division, U. S. Atomic Energy Commission. The paper was presented in part before the American Chemical Society, Atlantic City, Sept. '49.

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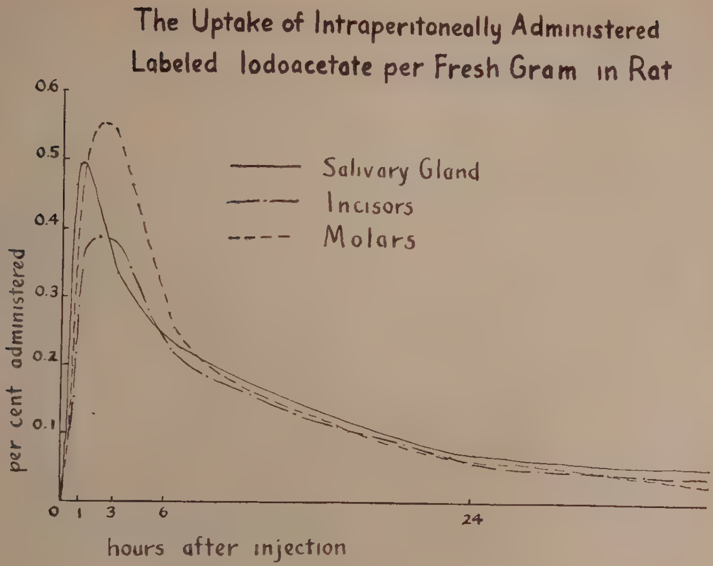

FIG. 1.

molecule. It was necessary to apply a mass absorption correction factor, as varying amounts of dried tissue were employed in the samples. In order to determine the degree of absorption of the radiation from I^{131} by varying amounts of mass, an aliquot of the dose was prepared and measured for activity with no mass present. Small amounts of water were added and after each addition a determination of the activity was made. From this a curve was derived giving the relationship between the mass of the sample and the percent of activity absorbed.

The same procedure was followed in the distribution studies. In this case, a total of 6 rats each were used for the iodoacetate and iodine studies, and the animals were sacrificed three hours after the intraperitoneal injections. The rats in the iodoacetate study received the same dosage as in the pilot study; the rats in the iodine study received a dosage with an activity of about 15 μc of I^{131}.

Results and discussion. The results of the preliminary study, shown in Fig. 1, indicate that the maximum uptake of labelled iodoacetate by the oral tissues occurs about 3 hours after injection. All further studies were therefore made at this time interval. Direct comparison of uptake of labelled iodoacetate and iodine by rat tissues after injection under comparable conditions is given in Table I. The range of individual variations in the rats was not great enough to alter the general findings.

The most striking effect shown in this table is the uptake of the two substances by the thyroid gland, as compared with the uptake by all the other tissues. Iodide is absorbed selectively, as expected, and the other tissues show only a small content of I^{131}. Uptake of iodoacetate, while higher in the thyroid than in the other tissues, is taken up to a much smaller degree than is iodide. In the other tissues, however, the content of iodoacetate is nearly twice that of iodide. In view of the fact that on standing, iodoacetate liberates some iodine, it is very possible that at all times a little iodine is present. If this is so, this free iodine will be taken from the blood by the thyroid, and may very possibly account for the higher values obtained in the thyroid with iodoacetate. The results observed in the other organs would then be due entirely to iodoacetate. It is noteworthy that in all organs except the thyroid, the uptake of iodoacetate is from 1.5 to 2 times as high as the

TABLE I. % of Administered Dose of Labelled Substance per g of Fresh Wt of Rat Tissues ± σ_m (Stand. Deviation of the Mean).

Tissue	Iodoacetate	I^{131}
Thyroid	8.304 ± .777	152.448 ± 10.542
Blood	1.201 ± .162	.698 ± .112
Saliva	.948 ± .265	——
Adrenals	.648 ± .159	.226 ± .057
Molars	.505 ± .109	.211 ± .031
Liver	.467 ± .078	.255 ± .033
Kidney	.402 ± .131	.266 ± .034
Incisors	.397 ± .138	.184 ± .017
Salivary glands	.358 ± .078	.250 ± .013
Skeleton	.285 ± .085	.187 ± .015
Striated muscle	.159 ± .065	.111 ± .031

uptake of I^{131}. This is probably due to the higher concentration of iodoacetate in the blood, almost double that of blood iodide, which is rapidly lowered by the thyroid. This fact, together with the rapid decrease in concentration with time seen in the oral tissues (Fig. 1) indicates that there is probably no selective uptake of iodoacetate. A diffusion into and out of the organs is indicated, a behavior characteristic also of iodine, where the thyroid is not involved.

Summary. (1) The distribution of iodoacetate and of potassium iodide, both labelled with I^{131}, has been determined in rats three hours after intraperitoneal injection. (2) The thyroid gland does not show a high selective uptake of iodoacetate. (3) Uptake of iodoacetate by tissues other than the thyroid is about twice that of iodide. There is no evidence of selective absorption by any tissue studied.

The authors wish to extend grateful thanks to Dr. Henry Elliott of the Department of Pharmacology, University of California Medical School for his valuable advice during this work, and for permitting the use of his Geiger-Muller Counter.

Received June 18, 1951. P.S.E.B.M., 1951, v77.

Response of Castrated Male and Female Hyperthyroid Rats to Vitamin B_{12}.*
(18869)

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Recent studies in this laboratory(1) indicated that the addition of vit. B_{12} to a purified ration containing toxic levels of iodinated casein effected a greater growth response in male than in female rats. Carrick(2) observed a sex difference in the response of chicks to fish solubles added to a ration presumably deficient in vit. B_{12}. An assay method for the determination of vit. B_{12} employing the hyperthyroid rat was developed by Frost *et al.*(3). These workers reported no sex difference in growth response to supplementation. Male and female offspring of vit. B_{12}-depleted rats showed a decided difference in growth response in studies reported by McCollum and Chow(4). Male rats grew more rapidly in the absence of vit. B_{12} supplementation than did their litter-mate females, suggesting a lower need for or more storage of this nutrient in the male rat. These results seemed to warrant further investigation of the influence of sex on the growth-promoting effect of vit. B_{12} in the hyperthyroid rat. Studies involving normal and castrate rats of both sexes were therefore initiated.

Materials and methods. Weanling rats of the Sprague-Dawley strain were used in this study. Thirty male and 18 female rats were

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* Published with the approval of the Director, Oklahoma Agricultural Experiment Station.

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3. Frost, D. V., Fricke, H. W., and Spruth, H. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 102.

4. McCollum, E. H., and Chow, B. F., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 20.

TABLE I. Composition of Rations.

Ingredient	Ration* 1 (%)	Ration* 2 (%)	Ration* 3 (%)
Corn starch	68.80	68.80	68.80
Soybean protein†	21.75	21.75	19.75
Corn oil	5.00	5.00	5.00
Mineral mixture‡	4.00	4.00	4.00
DL-methionine	.20	.20	.20
Iodinated casein§	.25	.25	.25
Liver concentrate powder, 1:20	—	—	2.00
Vit. B_{12}, .25 mg/kg	—	+	—

* Vitamin mixture was fed to supply the following amounts of water soluble vitamins per kg of ration: thiamin 6 mg, riboflavin 12 mg, niacin 40 mg, calcium pantothenate 60 mg, pyridoxine 6 mg, para-amino benzoic acid 100 mg, folic acid 5.0 mg, inositol 1 g, choline chloride 2 g. During standardization period only one-half of above amounts of various vitamins were fed. In addition to water soluble vitamins the rats received twice weekly 2 drops of mixture of cod liver oil and alpha tocopherol.

† Sodium proteinate, Archer-Daniels-Midland, Minneapolis, Minn.

‡ Hegsted, D. M. *et al.*, *J. Biol. Chem.*, 1941, v 138, 460.

§ "Protamone," Cerophyl Laboratories, Kansas City, Mo.

anesthetized with light ether anesthesia and the testicles and ovaries respectively removed. Thirty normal rats of each sex were retained as positive controls. All rats were fed ration 1 (Table I) without the addition of the thyroid-active material for a recovery and adjustment period of 10 days. At the end of this period, the rats were allotted into groups according to sex, weight, and gain during the standardization period. The rations shown in Table I were fed during the ensuing 2-week period; food and water were available *ad lib.* The rats were housed in individual cages and weighed at weekly intervals. Further details regarding the procedures employed will be found in a prevous communication(1).

Discussion. The results of this study are summarized in Table II. Examination of these data shows that the addition of either vit. B_{12} or 1:20 liver powder to the basal ration containing toxic levels of iodinated casein resulted in an increase of growth for all treatments. Analysis of variance of the data(5) showed that the differences were significant at the 1% level of probability. Normal males fed 1:20 liver powder showed an enhanced rate of growth compared with similar animals receiving crystalline vit. B_{12}. This difference was significant at the 5% level of probability. Liver also produced slightly better gains in castrate males than did vit. B_{12}. Normal females responded to either supplement, but significant differences between supplements were not found. Oophorectomized females responded in a similar manner as did normal males, but the differences in growth between supplements, although of nearly the same magnitude as in the normal males, did not achieve statistical significance.

It appears from these data that the male sex hormone may act synergistically in the hyperthyroid rat with some factor present in liver and not identical with vit. B_{12} or that the female sex hormone is antagonistic to such a factor. Thus, normal males and castrate females responded in a similar manner as did castrate males and normal females. The suggestion that factors other than B_{12} are necessary to cause a reversal of the growth-depressant effect of thyroid toxicity is in agreement with some of the findings of Ershoff(6). This worker, however, did not observe the growth stimulus with vit. B_{12} found in these experiments. The results of this study also suggest that the use of hyperthyroid animals for assay purposes without regard to sex, as recommended by Frost *et al.*(3), requires further investigation.

Summary. The growth response of normal and castrate hyperthyroid rats to supplementation with vit. B_{12} and liver concentrate powder has been investigated. Both supplements were effective in partially counteracting the growth depression induced by feeding toxic levels of iodinated casein to normal and castrate rats of both sexes. Vit. B_{12} was less effective than 1:20 liver powder for normal males and oophorectomized females. There were little difference in response to these supplements in normal females and castrate males. A sex difference in the growth response of the hyperthyroid rat to a factor

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TABLE II. Response of Male, Female, and Castrated Rats to Rations Deficient in Vit. B_{12}.

Ration	2 weeks' gain Uncastrated males, g	Castrated males, g	Uncastrated females, g	Castrated females, g
Ration 1 (basal ration)	46.7 (10)*	47 (10)	43.8 (10)	42 (6)
Ration 2 (ration 1 + 25 μg vit. B_{12} per kilo ration)	57.7 (10)†	60 (10)†	63 (10)†	64.4 (6)†
Ration 3 (ration 1 + 2% 1:20 liver concentrate powder)	69.2 (10)†‡	64.4 (10)†	62.2 (10)†	71.3 (6)†

* Numbers in parentheses are number of rats fed that ration.

† Indicates differences compared to basal ration were significant at 1% level of probability.

‡ Difference between gains of uncastrated males fed ration 3 and those fed ration 2 was significant at 5% level of probability.

present in liver concentrate and not identical with vit. B_{12} is suggested.

Appreciation is expressed to Merck and Co., Rahway, N. J., for the crystalline vitamins and vit. B_{12}, to Lederle Laboratories Division, American Cyanamid Corp., Pearl River, N. Y., for folic acid, to Wilson and Co., Chicago, Ill., for liver concentrate, and to the Cerophyl Laboratories, Kansas City, Mo., for iodinated casein (Protamone).

Received June 18, 1951. P.S.E.B.M., 1951, v77.

Metabolism of Plasma Substitute I. Dextran (Macrodex). (18870)

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The fate of dextran, unexcreted after intravenous infusion, is still undetermined. Bull and coworkers(1) report that it is not broken down by various animal tissues *in vitro*. Engstrand and Arberg(2) also have stated that dextran is not affected by tissue extracts, but is destroyed upon incubation with feces. They were able to recover 28% of infused dextran from the alimentary canal of the cat and 9% in the rabbit. We have attempted to confirm their results in 3 anesthetized dogs prepared as follows: (a) cannulae were placed in the bile duct and pyloric sphincter; (b) the upper end of the duodenum was tied off and a cannula placed in the lower end; (c) the upper end of the jejunum was tied off and a cannula placed in the ileo-caecal junction. The fluid from these areas was aspirated every 2 hours for 24 hours and each 2-hour sample was analyzed for dextran by the method of Hint and Thorsen(3). Less than 0.1% of 200 ml of 6% solution in physiologic saline infused, was found in any one of the regions cannulated. Failure to account for unexcreted dextran in the gastrointestinal tract led to study of the fate of this substance in the phlorizinized starved dog(4) to determine possible *in vivo* breakdown to dextrose.

Method. Mongrel dogs of both sexes were starved except for water. After 3 days, daily subcutaneous injections of 1 g of phlorizin suspended in 10 ml of olive oil, were begun and continued for the duration of the experiment (13-15 days). The animals were kept in metabolism cages and the urine collected every 24 hours. Complete collection was assured by catheterization. Dextrose in urine

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TABLE I. Changes in D/N Ratio after Infusion of 200 ml of 6% Solution of Dextran in Physiologic Saline.

Dog No.	Pre-infusion Avg	Range	24-48 hr post-infusion D/N
2	1.47	1.29-1.64	3.75
7	2.26	1.70-2.56	3.66
13	3.15	3.07-3.37	4.89
20	2.86	1.98-3.64	3.94
33	2.67	2.09-3.04	4.20
41	3.26	2.98-3.59	5.54*

* This figure represents the D/N ratio for 48-72 hr period following infusion.

was determined by the method of Fister(5). Dextran does not interfere with this test. The non-protein nitrogen was measured by micro-Kjeldahl digestion of a tungstic acid treated aliquot of the urine. When the dextrose/nitrogen (D/N) ratio became constant, (usually after 4-5 days of phlorizin), 200 cc of 6% dextran solution in normal saline (Macrodex) was infused intravenously, and studies were continued for 3 days.

Results and discussion. The results are summarized in Table I. In each instance there is a marked increase in the D/N ratio beyond the limits of the average value for the control period. In the starved animal, protein is being utilized as a source of energy by conversion to dextrose. When phlorizin is administered, resorption of the dextrose is prevented and a point of stabilization is reached where the ratio of dextrose in urine to non-protein nitrogen in urine becomes constant. If a substance is then administered that will alter nitrogen or glucose metabolism, the D/N ratio will be changed. The increase in D/N ratio 24-48 hours after the infusion of dextran, may be explained by one of the following mechanisms: (a) breakdown of dextran to glucose; (b) mobilization of glycogen to yield glucose; (c) conversion of the plasma protein to glucose.

Conant and coworkers(6), working with starved rats, showed that in 24 hours, liver glycogen decreased from 0.9% to 0.1%. Matthee(7), with phlorizinized rats and rabbits has shown that the glycogen very soon disappears from the liver. Lusk(8) has shown only 0.4 g liver glycogen in a meat-fed phlorizinized dog. It is therefore unlikely that the mobilization of glycogen could cause increase in D/N ratio in our phlorizinized animals.

The administration of dextran causes a decrease in plasma protein concentration(1,8). If the decrease of plasma protein is caused by dilution of the protein through the plasma expanding properties of the dextran(9), there should be no alteration in the D/N ratio. On the other hand, if this is a real loss of plasma protein, it should be metabolized similarly to the other tissue proteins and there should still be no change in the D/N ratio from the preinfusion value. It is concluded, therefore, that the change in D/N ratio we have measured in the phlorizinized dog following infusion of dextran, is due to the breakdown of dextran to dextrose.

Summary. Indirect evidence is presented to show that a portion of infused dextran ("Macrodex") is broken down to dextrose in the phlorizinized starved dog.

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Received June 19, 1951. P.S.E.B.M., 1951, v77.

Comparative Action of Some Dinitrophenols on Fungus Spore Respiration.* (18871)

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Respirational and metabolic reactions to nitrated phenols by various biological systems were reviewed by Edsall(1), Clifton(2), and von Oettingen(3). The authors indicate that nitrated phenols may exert either a significant stimulatory or inhibitory action, dependent upon the concentration applied. Field *et al.* (4), in comparing the stimulatory capacity of 6-cyclopentyl-, 6-cyclohexyl-, 6-methyl-2,4-dinitrophenol (DNOC) and 2,4-dinitrophenol (DNP) on yeast respiration found that concentrationwise their efficiency was in the order named, and the total excitatory effect exerted by each was essentially the same. Qualitative similarities for DNOC and DNP in stimulating as well as in inhibiting the respiratory intensity of yeast suspension were demonstrated by Field and Tainter(5).

Evidence is here presented in the form of dosage-response data to show that certain substituents, when introduced into the DNP molecule, materially influence the ability of the resultant derivative to inhibit the oxygen uptake by the spores of the strongly cellulolytic fungus, *Myrothecium verrucaria.* The similarity of the degree of poisoning effected by 12 of the 16 dinitrophenols† considered here is pointed out. Correlative relationships between chemical structure and biological response are indicated, with the limited number of derivatives available.

Method. The solubility characteristics of many of the nitrophenols considered here necessitated first a means whereby complete solution of each derivative was assured at every dosage level to be considered. Although many organic solvents proved suitable, methyl cellosolve proved most practical since it had no effect upon spore respiration at concentrations needed for solubilization. A requisite quantity of a given derivative, previously verified by melting-point determination, was dissolved in the organic solvent, then added to a quantity of the buffered nutrient solution and subsequently made to volume. The reverse procedure of adding nutrient solution to the organically solubilized nitrophenol derivative occasionally caused flocculation of the compound. A buffered nutrient media, pH 6.5, was composed as follows: NH_4NO_3, 3.0 g; KH_2PO_4, 2.59 g; K_2HPO_4, 2.21 g; $MgSO_4 \cdot 7H_2O$, 0.75 g; sucrose, 50 g; distilled water to 1,000 ml. Preliminary experiments with this same spore population indicated that the buffering capacity of this medium was sufficient to maintain a pH 6.5 for at least a 24-hour period. The freshly prepared spore suspensions used in each determination were made from 4-day-old cultures of the fungus grown at 30°C in Petri dishes containing Difco nutrient agar overlaid with a circle of Whatman No. 5 filter paper. The mineral salt and carbohydrate portions of this medium were the same as those used for preparing the spore suspensions. Spores were harvested by flooding the surface of the culture with nutrient solution and freeing the spores with a glass rod when necessary. This suspension was filtered through cheesecloth and subsequently adjusted to a population of 50×10^6 spores per ml. The cus-

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* The work reported in this paper was sponsored by the Departments of Air Force, Army and Navy under Contract N7onr-291, Task Order 27.

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† The nitrophenols used in this investigation were kindly supplied by Dr. F. B. Smith of the Dow Chemical Co. and by Mr. W. E. Craig of Rohm and Haas Co.

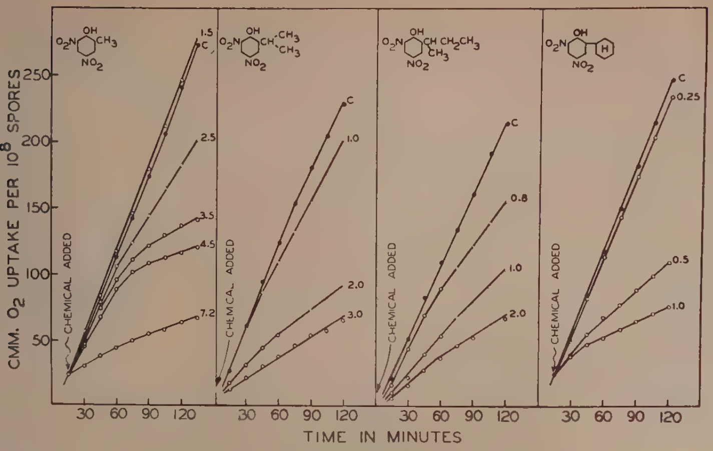

FIG. 1. Effect of several concentrations of 6-substituted 2,4-dinitrophenols on rate of oxygen uptake of a 10^8 spore population of *Myrothecium verrucaria*. Values at the ends of the curves indicate the number or fraction of 10^{-4} moles per liter final concentration and C indicates respiratory rate of untreated controls.

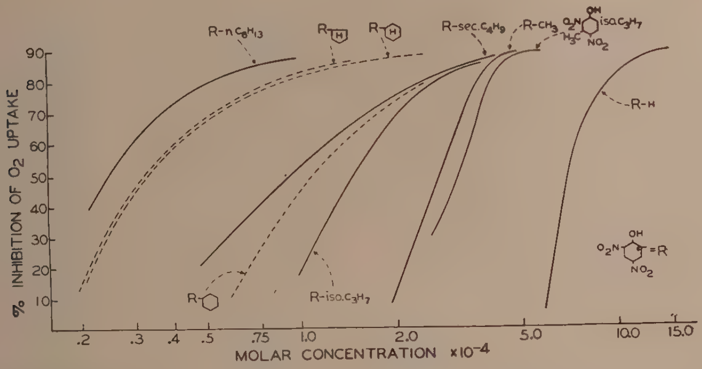

FIG. 2. Inhibitory effect of 6-C alkyl-, aryl- and cycloalkyldinitrophenols on oxygen respiration of *M. verrucaria* spores including the 2,4-dinitrophenol effect.

tomary manometric technics as described by Umbreit *et al.*(6), were applied to determine effects of the various nitrophenols on the oxygen uptake mechanism of the fungus spores.

Results. The time effect on oxygen uptake of several concentrations of four dinitrophenol derivatives included in this report is illustrated by the curves presented in Fig. 1. Generally, the behavior displayed here typifies the inhibitory action both as to its rapidity and type. In all cases with active derivatives the maximal effect on the oxidative mechanism is fully detectable within one hour after applying the poison. The action produced by DNOC was slightly retarded as compared with that caused by the other related chemicals here displayed. Inhibitory activity was calculated by comparing the slopes of the time-rate curves of the untreated controls with those of the treated spore suspensions, choosing slopes of the latter only when full poisoning had been effected. The comparative potency as well as the final common poisoning

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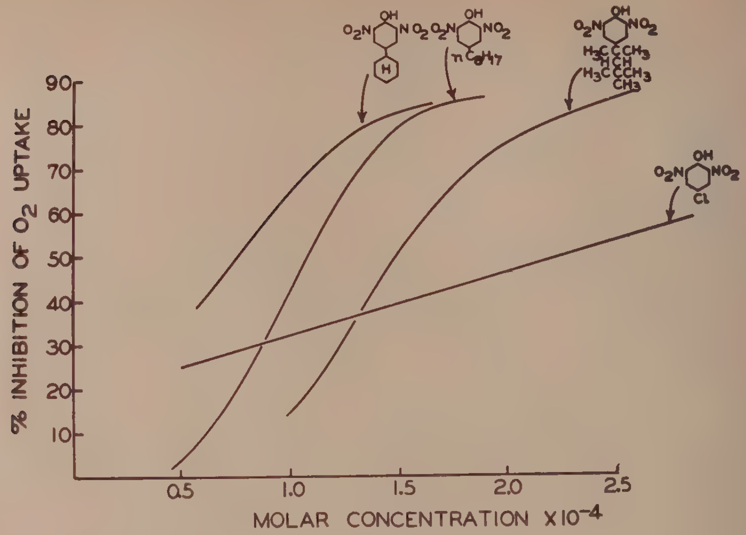

FIG. 3. Comparative inhibitory response of some 2,6-dinitrophenol derivatives on the respiration of *M. verrucaria* spores.

action of nine 2,4-DNPs on the oxidative mechanism of these fungus spores is shown by the dosage-response data in Fig. 2. Increase in the length of the alkyl chain of substituents located in the sixth position of the 2,4-DNP nucleus, generally increases effectiveness in reducing the rate of oxygen consumption, with the 6-*n*-hexyl-2,4-DNP molecule exercising the most toxic reaction.

Utilizing the ionization constant data as gathered by Krahl and Clowes(7), a calculation was made of the dissociated and undissociated concentrations for six of the derivatives included in the above figure. The calculations indicate that the responses shown in Fig. 2 are caused by the structural relationships rather than possible difference in undissociated concentrations formed by each derivative.

It is of interest to note that in the investigations by Field and Tainter(5) with yeast that the concentration causing 86 and 81% inhibition at pH 6.8 was 65 × 10^{-4} M for DNP and 15.2 × 10^{-4} M for DNOC. This indicates that the oxidative mechanism of these spores is significantly more sensitive to the poisoning action of DNP and DNOC than is that of the yeast cell. Generally similar inhibitory responses were reported by Newcomb(8) when investigating the effect of DNP levels ranging from 5 × 10^{-5} M to 2 × 10^{-4} M on the respiratory activity of tobacco callus tissue.

Inhibitory influences on the oxygen respiration of these fungus spores by some 2,6-DNP derivatives are indicated in Fig. 3. Except for the reactions demonstrated by the para-halogenated 2,6-DNP, they exerted a generally similar poisoning action as described for 2,4-DNP compounds. Altering the position of the cyclohexyl group to form *p*-cyclohexyl-2,6-DNP resulted in a slight loss of toxicity as compared with the isomeric 6-cyclohexyl-2,4-DNP (Fig. 2).

In contrast to the demonstrated effectiveness of the alkyl-, cycloalkyl-, and phenyl-dinitrophenols in poisoning the respiratory processes of spores of *Myrothecium verrucaria,* three derivatives formed by the introduction of a carboxyl, amino, or a third nitro group into the 2,4-DNP nucleus (resulting respectively in dinitrosalicylic, picramic, and picric acids) were relatively impotent in affecting this respiratory system (Fig. 4).

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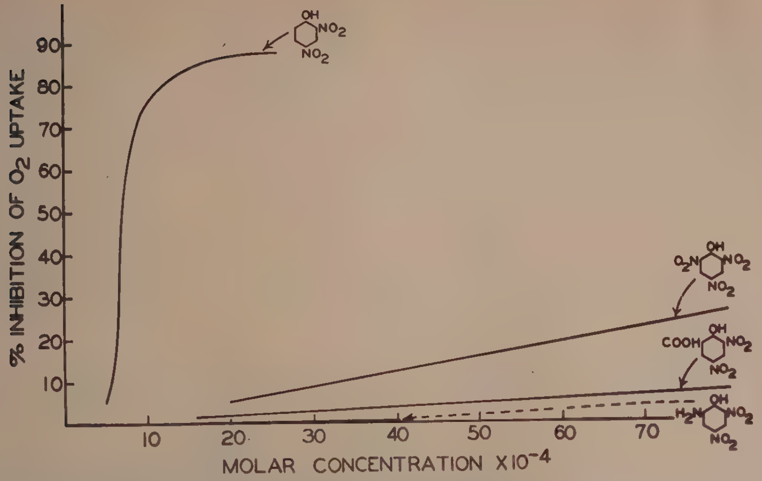

FIG. 4. Relative inhibitory effect on oxygen consumption of *M. verrucaria* spores of 2,4-dinitrophenol and compounds substituted in the 6-position with the nitro, carboxyl or amino group.

Summary. (1) The relative effects of 16 dinitrophenols in inhibiting the oxygen uptake mechanism of spores of *Myrothecium verrucaria* were studied. (2) The alkyl-, cycloalkyl-, or aryl- groups substituted ortho to the hydroxyl group in the 2,4-DNP molecule significantly increased the poisoning effect on this oxidative mechanism as compared to 2,4-DNP. (3) Effective concentrations of all active compounds manifested their full effect within an hour following contact with the spores. (4) The inhibitory effect as caused by all the alkyl substituted 2,4- and 2,6-DNPs averaged 85-90%. (5) Concentrationwise, the 6-*n*-hexyl-2,4-DNP compound proved the most potent, while dinitrosalicylic, picramic, and picric acids were significantly ineffective.

Received June 21, 1951. P.S.E.B.M., 1951, v77.

Primary Amide Groups of Human Hemoglobin.* (18872)

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Pauling *et al.*(1) observed that the hemoglobin isolated from patients with sickle cell anemia possesses a higher isoelectric point (IEP) than that from normal individuals. Schroeder *et al.*(2) have shown that the amino acid composition of the two proteins is almost identical, and postulated that differences in the folding of the peptide chains were responsible for the electrophoretic results.

An increase in the number of primary amide groups of the sickle cell hemoglobin with a consequent decrease in free carboxyl groups might also be responsible for the higher IEP of this protein. The data of Table I, however, indicate that the number of primary amide groups in normal hemoglobin and in hemoglobins isolated from patients with sickle

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* Aided by a research grant from Armour and Co.

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TABLE I. Number of Primary Amide Groups per Mole of Human Hemoglobin.

Non-sickle cell anemia hemoglobin source	Primary amide N, residues/mole	Sickle cell anemia hemoglobin source	Primary amide N, residues/mole
Mr. L. (polycythemia vera)	26	Miss S.	28.7
	22.9		25.9
	26		24.4
" M. (normal)	26.1		26.3
	25.8		26.7
	26.4	" P.	26.8
	23.5		26.8
	23.9		25.8
" C. (normal)	25.9	" B.	26.1
	25.9		26.5
Mrs. R. (acute rheumatic fever)	26.8		25.4
	26.8		
Miss T. (subacute bacterial endocarditis)	26.3		
	27.3		
Mrs. Q. (pulmonary infarction)	26.1		
	26.1		
Avg	25.7	Avg	26.3
Standard deviation = 1.22		Standard deviation = 1.15	

cell anemia, polycythemia vera, acute rheumatic fever, subacute bacterial endocarditis, and pulmonary infarction is essentially constant. An average of 26 primary amide groups per mole was obtained for all the hemoglobin samples analyzed.

Sources of hemoglobin. The group of 6 controls included 2 normal samples and one each from patients with the following diseases: Polycythemia vera, subacute bacterial endocarditis, pulmonary infarction, and acute rheumatic fever.[†] The experimental samples were obtained from 3 sickle cell anemic patients.[‡]

Isolation and analysis of hemoglobin. Solutions of carbon monoxide hemoglobin were prepared according to the method of Drabkin(3). They were stored under carbon monoxide at 4° until analysis. Hemoglobin concentration was determined with a Coleman Jr. spectrophotometer at 545 mμ(4).[§]

Determination of primary amide groups. A 1.00 ml aliquot containing approximately 8 mg of hemoglobin was pipetted into a test tube calibrated at 5.0 ml, then 4 ml of 3 N HCl was added. The tube was covered with a marble and placed in a boiling water bath for 3 hours. The ammonia liberated by this treatment was determined on an aliquot of this solution by microdiffusion in Conway vessels(5).

Results. The data, calculated as residues of primary amide groups per mole of hemoglobin, are set out in Table I. A value of 66,700 was used for the molecular weight of human hemoglobin(6).

Discussion. Schroeder *et al.*(2) found 36 residues of ammonia per mole in hemoglobin from normal individuals and 33 residues per mole in hemoglobin from patients with sickle cell anemia. These results are consistent with the values for the primary amide content reported here. Both groups of analyses indicate a constancy in the number of primary amide groups of the two proteins. The difference in the isoelectric points of the two proteins apparently is due to more subtle factors than the total number of amino acid residues or content of primary amide groups. Alteration in the sequence of the amino acids in the peptide chains is a possibility which should be considered in understanding this interesting problem.

[†] We wish to than Dr. George Cartwright, Department of Medicine, University of Utah, for obtaining these samples for us.

[‡] We wish to thank Dr. Carl Moore, Department of Medicine, George Washington University School of Medicine, for sending these samples to us.

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Summary. The hemoglobin from sickle cell anemic individuals does not differ significantly in primary amide content from normal hemoglobin. Normal human hemoglobin was found to contain 26 primary amide groups per mole.

Received June 22, 1951. P.S.E.B.M., 1951, v77.

Choline Requirement of Rats as Influenced by Age, Strain, Vitamin B_{12} and Folacin.* (18873)

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It was shown(1-3) that there was considerable variation in the minimum choline requirement of rats from different strains, sex, litter, and age. Copeland(3) noted that hooded rats of the Alabama Experiment Station strain (AES) had a much higher choline requirement for protection against renal hemorrhage than did albino rats of the Wistar strain. Since rats of the AES strain develop a high incidence of neoplasms(4-6) when subjected to chronic choline deficiency, it was of interest to investigate the choline requirement of the albino Sprague-Dawley (SD) strain as compared to the AES strain. The reports(7-9) that young rats of the Wistar strain are capable of synthesizing sufficient methyl and choline or of utilizing methanol for choline synthesis for growth and protection against renal damage prompted the investigation as to whether age and strain difference would influence these mechanisms under the experimental conditions employed in this laboratory.

Experimental. Weanling rats (21 days) of the SD and the AES strains weighing 40-45 g were placed in individual cages and uniformly grouped with respect to number, weight, sex, and litter. Feed and water were supplied *ad lib.* The basal diet contained extracted peanut meal 30 (50% protein)(10), sucrose, 39.5, extracted casein 6(11), salt mixture (undried) 4.4(11), L-cystine 0.1, cod liver oil 1, and lard 19. Vitamins were added (mg per kg of diet) as follows: Thiamine 2, pyridoxine 2, riboflavin 4, calcium pantothenate 10, niacin 20, i-inositol 200, alpha-tocopherol 25, alpha-tocopherol acetate 25, and 2-

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* Published with the approval of the Director of the Alabama Agricultural Experiment Station. A preliminary report of part of this work was given at the 118th National Meeting of the American Chemical Society, Sept. 8, 1950. This study was supported in part by grants from the American Cancer Society upon the recommendation of the Committee on Growth of the National Research Council, and from the National Cancer Institute of the National Institutes of Health, Public Health Service. Donations of crystalline vit. B_{12} and other vitamins were made by Merck and Co., and of folacin (pteroylglutamic acid) by Lederle Laboratories.

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TABLE I. Choline Requirement of Sprague-Dawley Rats for Protection Against Hemorrhagic Kidneys and for Lipotropism as Influenced by Vit. B_{12} and Folacin (8 Rats per Treatment).

Dietary supplement				Avg liver analyses		
Choline, %	Vit. B_{12}* and folacin	Avg wt gain, g/2 wk	Incidence of renal damage, %	Fat dry basis, %	Moisture, %	Choline per g dry liver, mg
.04	0	35	63	55.3	53.1	3.2
.08	0	58	25	52.3	52.4	3.9
.10	0	69	0	40.3	59.7	4.3
.20	0	73	0	19.5	66	5.8
.30	0	68	0	15.8	67.8	7.2
.40	0	75	0	12.9	67.8	7.8
.04	+	71	0	55.2	52.5	3.7
.08	+	75	0	43.3	61.3	4.3
.10	+	70	0	33.3	62.6	6.4
.15	+	75	0	15.3	67.9	7.7
.20	+	81	0	12.8	69	8.5

* Vit. B_{12} = 30 μg, and folacin = 2 mg/kg diet.

methyl-1,4-naphthoquinone 5. Where indicated, folacin was added at 2 or 10 mg and vit. B_{12} at 30 or 150 μg per kg of diet. The total methionine and choline contents of the diet were 0.30% and 0.005%, respectively.

All rats were necropsied after death or at the end of the 14-day experimental period; kidneys were examined for gross pathological lesions; and livers were analyzed for moisture, fat, and choline. Liver fat was expressed as per cent total ether-extractable material on a dry weight basis. Choline was determined by the method of Engel(12) with the following modifications: The sample was continuously extracted for 48 hours with boiling methanol rather than the 3 separate extractions totaling 24 hours; normal propyl alcohol was substituted for ethyl alcohol as a wash solution for the choline reineckate.

Results. The results of supplementing the basal diet for the SD strain of rats with varying levels of choline chloride with and without vit. B_{12} and folacin are given in Table I. Supplementation of the diet with vit. B_{12} and folacin markedly suppressed the accumulation of fat in the livers, reduced the incidence of renal damage, and increased the choline content of the livers. The liver fat of the rats fed the basal diet supplemented with 0.15% and 0.20% choline chloride plus vit. B_{12} and folacin was equal to that of rats receiving 0.30% and 0.40% choline chloride respectively, without vit. B_{12} and folacin. In the absence of dietary vit. B_{12} and folacin, the incidence of kidney hemorrhage was 67% and 25% when the choline chloride level was 0.04% and 0.08%, respectively. No renal damage occurred when the diet was supplemented with 0.04% choline chloride, and vit. B_{12} and folacin.

The choline concentration of the livers of rats receiving 0.10% choline chloride was increased an average of 49% (*i.e.*, from 4.3 to 6.4 mg per g dry liver) by the additional dietary supplement of vit. B_{12} and folacin.

The influence of age, strain, and dietary methanol supplements on the pathology of acute choline deficiency in the presence of adequate vit. B_{12} and folacin is shown by the data in Table II. Under these experimental conditions, both strains of rats failed to develop hemorrhagic kidneys when they were maintained on an adequate stock diet until 42 days of age prior to receiving the experimental diet. Liver fat of the SD strain rats was 33% as compared to 62% for the AES strain rats.

The incidence of renal hemorrhage and mortality was higher in the AES strain of rats than in the SD strain. When placed on experiment at 35 days of age, the AES strain rats had an incidence of 63% renal hemorrhage and 13% mortality. The incidence for SD strain rats was 12% and 0, respectively. Supplementation of the diet with 1% methanol was without apparent effect either for protecting against renal damage or for lipotropism.

Discussion. The experiments of this study

TABLE II. Effect of Age on Body Wt Gain, Kidney Damage, Mortality and Liver Fat of AES* and SD† Strain Rats‡ (8 Rats per Treatment).

Age at§ start of exp., days	Avg wt gain of survivors, g/2 wk AES	SD	Incidence renal damage, % AES	SD	Mortality, % AES	SD	Avg liver fat (dry basis), % AES	SD
21	16	39	100	100	38	38	55	—
28	35	54	100	63	38	0	67	65
35	53	61	63	12	13	0	64	66
42	58	69	0	0	0	0	62	33
35‖	56	70	50	50	25	0	64	65
42‖	—	66	—	0	—	0	—	26

* Alabama Experiment Station strain of rats (hooded).

† Sprague-Dawley strain of rats (albino).

‡ Basal diet supplemented with 150 μg vit. B_{12} and 10 mg folacin/kg.

§ The animals were maintained on an adequate stock diet from weaning (21 days) until the start of the experiment.

‖ 1% methanol added to the diets.

were conducted concurrently with those reported by Strength *et al.*(13) in which the AES strain of rats was used. These rats, when fed the basal diet supplemented with 0.04% choline chloride, developed renal damage, whereas only 63% of the SD strain rats had renal damage (Table II). In the absence of dietary vit. B_{12} and folacin, liver fat of the AES strain rats(13) was 12.3% as compared to 19.5% for SD strain rats (Table I), when the diet was supplemented with 0.20% choline chloride. At the 0.10% level of choline chloride plus vit. B_{12} and folacin, liver fat of the SD strain rats was 33.3% (Table I) as compared to 17.5% for the AES strain rats (13). At the 0.08% level of choline chloride plus vit. B_{12} and folacin, liver fat of the SD strain rats was 43.3% (Table I) as compared with 27.0% for the AES strain rats(13). These results indicate that, although weanling rats of the SD strain are apparently less susceptible to renal damage than AES strain rats, they have an initial higher choline requirement for maintenance of normal liver fat.

Supplementation of the diet with vit. B_{12} and folacin markedly suppressed the accumulation of fat in the livers of rats fed subprotective levels of choline. Under these conditions the minimum choline requirement for SD strain rats for lipotropism was 0.40% in the absence of vit. B_{12} and folacin and 0.20% in the presence of these factors. The concentration of choline in the liver parallels the removal of fat from the liver. Rats of the SD strain fed vit. B_{12} and folacin plus 0.15% choline chloride had liver choline values equal to those of rats fed 0.40% choline without vit. B_{12} and folacin.

Forty-two-day-old rats of either the AES or SD strain when fed the basal diet supplemented with vit. B_{12} and folacin grew rapidly and failed to develop renal hemorrhage even in the absence of a choline supplement. This indicates that animals of this age are capable of synthesizing sufficient choline and perhaps utilizing it more efficiently than weanling rats. The liver fat values indicate that 42-day-old SD strain rats are apparently able to synthesize and perhaps utilize choline more efficiently than AES strain rats when fed a diet supplemented with vit. B_{12} and folacin. This is a striking contrast with the results obtained with weanling rats of these strains.

Under the experimental conditions employed, the addition of methanol to the diet afforded no apparent protective action against renal hemorrhage.

Summary. (1) Weanling rats of the Sprague-Dawley (SD) strain have a lower choline requirement for protection against renal hemorrhage and a higher requirement for maintenance of normal liver fat than was observed in rats of the Alabama Experiment Station (AES) strain. (2) Forty-two-day-old rats of either strain grew rapidly and failed to develop hemorrhagic kidneys when they were fed the choline-deficient basal diet

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supplemented with only vit. B_{12} and folacin. However, liver fat of the SD strain rats was 33% (dry basis) as compared to 62% for the AES strain rats. (3) The addition of vit. B_{12} and folacin to the basal diet supplemented with suboptimal choline chloride markedly suppressed the accumulation of fat in the liver, reduced the incidence of renal damage and increased the choline content of the livers of the SD strain of rats. Liver fat values averaging 12.8% (dry basis) were obtained with a 0.20% choline chloride supplement in the presence of vit. B_{12} and folacin as compared to a requirement of 0.40% choline chloride in the absence of vit. B_{12} and folacin.

Received June 25, 1951. P.S.E.B.M., 1951, v77.

Reduction of X-ray Sensitivity of *Escherichia coli* B/r by Sulfhydryl Compounds, Alcohols, Glycols, and Sodium Hydrosulfite.* (18874)

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It has been shown(1) that oxygen has a marked effect on the survival of X-irradiated *Escherichia coli* B/r. A striking increase in survival was attained by saturating the bacterial suspensions with nitrogen before exposure to X-radiation. In this study, the X-ray sensitivity of *E. coli* B/r in the presence of 2,3 dimercaptopropanol (BAL), sodium hydrosulfite, and ethanol has been examined.

Suspensions of *E. coli* B/r cultured aerobically 20-24 hours in nutrient broth (Difco) were washed and resuspended in 1/15 M phosphate buffer. A 0.1 ml portion containing about 10^{10} cells was introduced into each of several 2 ml test tubes together with 0.9 ml of the test compound in phosphate buffer. Buffer solutions 0.04 M in sodium hydrosulfite and solutions 3.5 M in ethanol were used in examining the protective ability of these compounds. Dispersions equivalent to 0.04 M BAL were prepared by shaking in buffer a short time before addition to the suspensions. These were shown in other experiments(2) to be the maximally effective concentrations of these agents.

The diluted suspensions were cooled to 2°C and maintained at this temperature during the exposure to X-radiation. The source of X-rays was a Maxitron 250 unit operated at 250 kvp at 30 ma (3 mm added aluminum filter). The test tubes were held in a slanting position, at a 35° angle from the horizontal, under the X-ray tube by a circular lucite holder containing ice and ice water. The irradiated suspensions were diluted with nutrient broth and aliquots transferred to several broth agar plates, the average number of colonies developing in 24 hours at 37°C being used to calculate the number of surviving cells.

The log of the survival of *E. coli* B/r grown under aerobic conditions is a linear function of the X-ray dose, at least over the range 15,000 to 90,000 r. This relationship is illustrated in Fig. 1 for untreated suspensions and for suspensions which have been bubbled for 15 minutes with nitrogen immediately before the exposure period. As shown in Fig. 1-2, a similar relationship also obtains in the presence of BAL, sodium hydrosulfite, and ethanol.

The protective action of a chemical agent can be expressed simply as the ratio of the X-ray dose required to kill a given number of cells in the presence of the compound to that required in the absence of the compound. This method of presenting data is essentially that used by Kelner(3). "Isoefficiency ra-

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* Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

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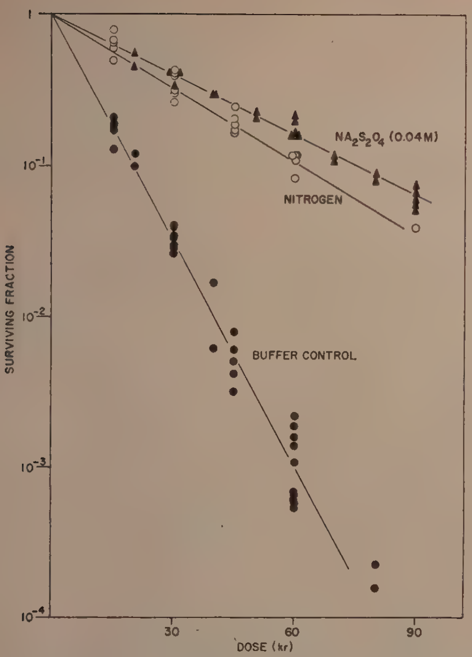

FIG. 1. Effect of nitrogen saturation and sodium hydrosulfite on survival of *E. coli* B/r suspensions X-irradiated at 2°C.

tios" calculated from data presented graphically in Fig. 1-2, are given in Table I.

The "isoefficiency ratio" for BAL, which has 2 sulfhydryl groups, is about twice that found for other compounds which have only one sulfhydryl group, *e.g.*, cysteine, mercaptosuccinic acid, mercaptopyruvic acid, and 2(2-mercaptoethoxy) ethanol using equivalent molarities. It might be considered that the sulfhydryl compounds have the ability to protect the enzyme systems in bacterial cells, since Barron(4) has shown that cysteine and glutathione protect X-irradiated enzyme substrates. However, the entrance of these compounds into the bacterial cell has not been shown. The protective action of cysteine in mice under certain conditions has been demonstrated by Patt(5).

Because sodium hydrosulfite is a strong reducing agent and combines rapidly with molecular oxygen in aqueous solution, its mode of action may be simply a removal of oxygen from the bacterial suspensions. Reference has already been made to the finding in this laboratory that a reduction in the oxygen tension of *E. coli* B/r suspensions before X-irradiation increases the survival. It may be speculated that the removal of molecular oxygen, either by a chemical agent or by bubbling an inert gas through the suspension, interferes with the production of highly reactive radiodecomposition products(6-13).

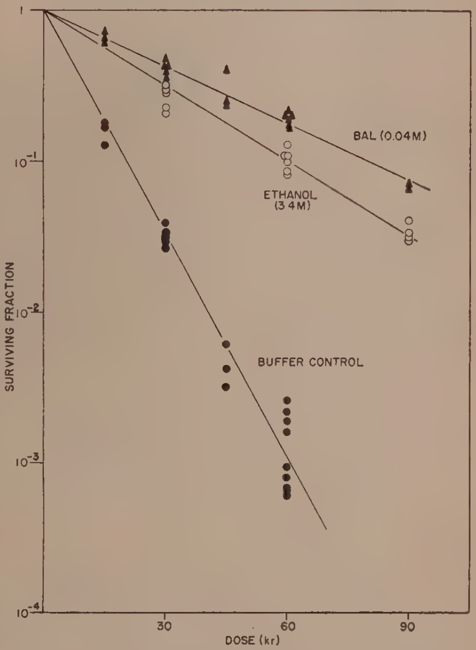

FIG. 2. Effect of ethanol and BAL on survival of *E. coli* B/r suspensions X-irradiated at 2°C.

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TABLE I. Isoefficiency Ratios* for Nitrogen, BAL, Ethanol, and Sodium Hydrosulfite Calculated from Data Presented in Fig. 1 and 2.

	Nitrogen	BAL (0.04 M)	Ethanol (3.5 M)	Sodium hydrosulfite (0.04 M)
Dose ratio ($\rho = 10^{-1}$ survival)	3.2	4.0	3.1	3.7

$$* \text{ Isoefficiency ratio} = \frac{\text{kr required to give } \rho \text{ survival in presence of agent}}{\text{kr required to give } \rho \text{ survival without agent}}.$$

However, since the "isoefficiency ratio" for sodium hydrosulfite is higher than that found by saturating the suspension with nitrogen, it seems worth while to further investigate the protective action of sodium hydrosulfite.

Methanol; 2-propanol; 1-butanol; 2-methyl-1-propanol; 2-methyl-2-propanol; 1,2,3-propanetriol; 1,2-propanediol; and 1,2-ethylenedioxydiol are about as effective as ethanol on a molar basis.

The concentration of ethanol necessary to effect the large increase in cell survival of *E. coli* B/r (Fig. 2) is much higher than the concentrations reported to interfere with the X-ray inactivation of trypsin(14). However, ethanol in lower concentrations (0.02-0.1 M) has been found to increase cell survival if the suspensions are incubated in its presence at higher temperatures (25-37°C) prior to irradiation.

Summary The X-ray sensitivity of *E. coli* is reduced if the cells are irradiated in the presence of certain chemical agents. BAL, ethanol, and sodium hydrosulfite increase the dose required to kill a given number of cells in suspensions at 2°C by a factor of 3-4. Other sulfhydryl compounds, glycols, and low-molecular-weight alcohols also increase the survival of *E. coli* suspensions.

The authors are indebted to Dr. C. T. Bahner, Carson-Newman College; Mr. L. E. Klein, Monsanto Chemical Company; and Drs. D. G. Doherty and A. H. Doermann, Biology Division, Oak Ridge National Laboratory for valuable suggestions. The technical assistance of Mr. A. W. Burke is acknowledged.

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Received June 25, 1951. P.S.E.B.M., 1951, v77.

Maintenance of Normal Thyroid Activity after Transplantation of Thyroid Gland into Spleen or Kidney. (18875)

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Shortly after the intravenous injection of thyroxin labelled with radioactive iodine, much of the radioactivity can be demonstrated in the liver, gastrointestinal contents and feces(1,2). Most of the gastrointestinal and fecal iodine is no longer in the form of thyroxin(1). Since thyroid hormone appears to circulate in the form of thyroxin(3) it might be concluded that the liver normally plays an important part in the isolation, destruction and excretion of circulating thyroid hormone. However, the sudden introduction of a test material into the bloodstream may cause a transient elevation in the concentration of the

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substance, and thus call into play mechanisms of catabolism not ordinarily involved in the metabolism of normal concentrations of the compound. The following experiment was therefore designed to determine whether the liver plays any important role in the catabolism and excretion of endogenous thyroid hormone secreted at a normal rate.

Method. Three groups of Sprague-Dawley rats were thyroidectomized under ether anesthesia. In one group, the right lobe of the excised gland was implanted deep in the spleen. In a second group, the thyroid implant was made in the right kidney. The third group served as a thyroidectomized control. A fourth group was not operated on.

The animals were maintained on Rockland rat ration. The operated animals received drinking water fortified with 9 g calcium lactate and 1 g calcium chloride per liter. The rats were weighed weekly.

After 82 days, the animals were anesthetized with intraperitoneal pentobarbital. The abdomen was opened and as much blood as possible was withdrawn from the inferior vena cava into an oxalated syringe. The oxalated blood was centrifuged, and the protein-bound iodine in the plasma determined by the alkaline ash method of Barker and Humphrey(4).

The thyroid glands were removed from the normal animals and placed in Zenker's fixative. The implants were identified in kidney or spleen and excised in a small tissue block which was similarly fixed. The height of thyroid cells was estimated with an ocular micrometer on the hematoxylin-eosin stained sections. By the use of a mechanical stage, random follicles were selected in a progressive pattern which prevented recounting the same follicle. Four cells were measured in each follicle, the cells selected for measurement being distributed equally about the circumference of the follicle. At least 20 cells were measured in each section.

Observations. Transplantation of the thyroid into the spleen was accompanied by a fairly high immediate operative mortality, but all of the animals which survived the immediate postoperative period lived until the end of the experiment. It is unfortunate that only 3 spleen-transplanted animals survived; however, the results obtained were so clear cut that fairly reliable conclusions are possible.

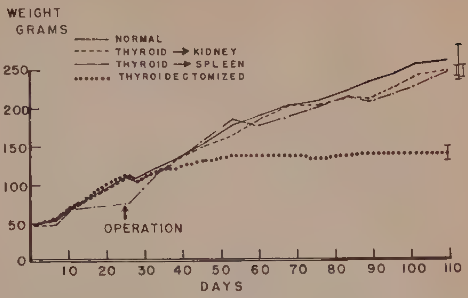

FIG. 1. Comparison of growth curves of normal, thyroidectomized and thyroid-transplanted male rats. Vertical lines at 110 days define standard error of mean of terminal weights.

Transplantation of the thyroid gland to spleen or to kidney had no detectable effect on the rate of growth of the animals (Fig. 1) or on their final weight (Table I). Thyroidectomy, as expected, caused almost complete cessation of growth.

The splenic implants maintained a normal plasma protein-bound iodine concentration. The kidney-implanted animals had a somewhat reduced protein-bound iodine concentration as compared to the unoperated controls ($t = 3.53$; $p < .01$), but it is doubtful whether the degree of depression can be considered physiologically significant. It was impossible to obtain enough blood to do the protein-bound iodine analysis in the case of the thyroidectomized rats because of their small size. In a group of five full-grown rats of the same strain, which had been thyroidectomized three weeks prior to bleeding, the plasma protein-bound iodine was significantly lower than in any of the groups with thyroid tissue present ($p < .01$).

From these data it is apparent that the thyroid transplants in both kidney and spleen were functional, and that they were able to maintain the animals in a euthyroid state, as manifested by normal growth curves and normal plasma protein-bound iodine concentra-

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TABLE I. Effect of Thyroid Transplant and Thyroidectomy Upon Growth and Plasma Protein-Bound Iodine of Male White Rats.

Procedure	No. rats	Preoperative wt, g	Terminal wt, 82 days post-operative, g	Total gain, g	Plasma protein-bound iodine, $\mu g/100$ ml	Mean thyroid cell height, μ
Normal	5	90 ± 18.2*	248 ± 8.6	158 ± 14.2	6.5 ± .37	10.9 ± 1.9
Thyroidectomized	5	104 ± 10.7	140 ± 9.7	36 ± 10.2	3 ± .22†	
Thyroid to spleen	3	114 ± 6.3	263 ± 17.6	149 ± 13.2	6.2 ± .21	11.7 ± .3
Thyroid to kidney	6	109 ± 4.5	249 ± 9.8	140 ± 7.6	4.7 ± .35	12.8 ± .5

* All values given are mean ± standard error of the mean $(\sqrt{d^2/n(n-1)}$.

† PBI determinations were impossible on the thyroidectomized rats of this series because sufficient plasma could not be obtained. The values in the table refer to adult thyroidectomized rats of the same strain.

tions. If the liver were normally concerned with the destruction of thyroxin, one would expect the spleen-implanted animals to show signs of thyroid deficiency. This might not have occurred, however, even in the face of hepatic destruction of thyroid hormone, if the splenic implant were sufficiently active to flood and overwhelm the enzyme systems concerned with thyroxin degradation in the liver. If this were the case, one would expect to see signs of increased secretory activity in the splenic implants. Examination of the implants, however, failed to demonstrate any morphologic abnormality of the thyroid cells; and, the mean follicular cell height was not increased in the thyroid tissue implanted in the spleen (Table I).

Discussion. These observations indicate that when thyroid hormone is secreted directly into the portal vein, and therefore must pass the liver before reaching the general circulation, no significant impairment of effectiveness of the hormone can be detected. This might simply mean that the site of action of the hormone lies in the liver, and that the "detoxification and excretion" previously discussed is a reflection of the normal utilization of the hormone. Since the effects of thyroxin appear to be manifested in all tissues of the body, however, this seems unlikely. If the site of action of thyroxin is in the peripheral tissues, the liver seems not to interfere with the passage of the hormone from spleen to its site of action.

These observations appear to contradict the results obtained when labelled thyroxin is injected. The difference is probably due to the fact that the injection of thyroxin, even in very small quantities, temporarily raises the concentration of thyroxin in the blood. Under these circumstances, the injected thyroxin may be handled in part as a foreign substance. This explanation is favored by the observation of Gross and LeBlond that as the dose of thyroxin was decreased in the labelled-thyroxin experiments, smaller and smaller fractions of the radioiodine were recovered in the liver plus gastrointestinal tract plus feces(2). The fact that orally administered thyroxin is as effective calorigenically as intravenously administered hormone(5) also implies that the thyroid hormone is not destroyed by the liver when the quantities involved are small.

Summary and conclusions. Thyroid fragments implanted into the spleen of thyroidectomized rats are functional and capable of maintaining normal growth and plasma protein-bound iodine concentrations. No morphologic evidence of hyperactivity of the implants could be observed. It therefore appears that the liver does not destroy and excrete the thyroid hormone to a significant extent, when the hormone is present in physiologic quantities.

Statistical analyses were carried out as described by Snedecor, G. W., Statistical Methods. Iowa State College Press, 1946.

The author is grateful to Miss Lillian Evans for making the histological sections, to Dr. Abner Golden for his help in interpreting them, and to Mrs. Jean A. Estes, Mrs. Joan K. Medwedeff and Mrs. Mary Ann Hagewood for their assistance.

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Received June 26, 1951. P.S.E.B.M., 1951, v77.

Further Studies on Thonzylamine in Treatment of Experimental Tuberculosis. (18876)

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Since many of the symptoms of tuberculosis are believed to result in part from hypersensitivity to the products of the tubercle bacillus(1), we thought it worthwhile to study the effect of an antihistamine on the course of tuberculosis in guinea pigs.

In a preliminary experiment(2) we found that thonzylamine (Neohetramine) reduced the extent of tuberculosis in guinea pigs sacrificed 3 months after infection. In the following paragraphs we report the results of an experiment designed to determine the effect of thonzylamine alone and in combination with non-curative doses of streptomycin on the survival time of tuberculous guinea pigs.

Materials and methods. 160 Tuberculin-negative male guinea pigs, weighing 300-400 g were infected subcutaneously in the region of the groin with *Mycobacterium tuberculosis,* strain H 37 RV, each animal receiving 0.1 mg of bacilli (moist weight) per 500 g body weight. They were then divided into 4 groups as follows: Group 1—60 control guinea pigs received 0.5 ml physiological saline subcutaneously twice daily at 9 A.M. and 5 P.M. Group 2—60 guinea pigs received 6 mg thonzylamine dissolved in 0.5 ml physiological saline twice daily as above. Group 3—20 guinea pigs received 6 mg thonzylamine and 1 mg streptomycin dissolved in 0.5 ml physiological saline twice daily as above. Group 4—20 guinea pigs received 1 mg streptomycin dissolved in physiological saline twice daily as above.

Treatment, started on the day of infection, was continued until the death of the animal. Two months after the experiment was started, the dose of thonzylamine was increased to 9 mg twice daily for all animals receiving that drug.

All guinea pigs were weighed weekly, and tuberculin tests were performed every 7-10 days with varying amounts of O.T., by the Mantoux method. Each animal was autopsied as soon as possible after death, and the extent of tuberculosis in lungs, liver, spleen and lymph glands was scored as previously described(2). Representative sections of these 4 tissues, selected at random, were fixed in Zenker's fluid and examined microscopically. At no time did the examiner know the treatment undergone by the animal being autopsied, or the source of the material under microscopic examination.

Results. Tuberculin tests. At no time during the experiment did the treated animals react differently to intracutaneous O.T. than did the controls.

Weight. Examination of average weight curves disclosed no appreciable difference between the growth rates of the animals in groups 1, 2, and 3, although they suggested animals in group 4 gained more weight in the later stages of the experiment than the others. However, the percent change in weight between the beginning and the end of the ex-

TABLE I. Wt in g.

	At beginning of exp.		At end of exp.		
Group	No. animals	Avg wt	No. animals	Avg wt	% change
1	(60)	328	(9)	783	+239
2	(60)	326	(14)	782	+240
3	(20)	412	(13)	757	+184
4	(20)	399	(12)	924	+231

TABLE II. Analysis of Survival Data.

Group	1	2	3	4
Mean survival time, days	175.9	189.4	235.4	219.6
S $(y-\bar{y})^2$	216,787	155,433	57,059	110,628
n	56	58	19	20
t	(1 vs 2) 2.5137	(3 vs 4) 1.55		(1 vs 4) 5.9321

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TABLE III. Average Gross Autopsy Scores of Streptomycin Treated, Thonzylamine Plus Streptomycin Treated, Thonzylamine Treated, and Control Tuberculous Guinea Pigs.

Group	Treatment of	No. Guinea pigs	Lungs	Liver	Spleen	Glands	Summary
4	Streptomycin	20	1.1	1	1.5	1.9	5.5
3	Thonzylamine + Streptomycin	19	1	1.1	1.7	1.6	5.4
2	Thonzylamine	58	1.6	1.5	1.9	1.6	6.6
1	Physiological saline	56	1.7	1.8	2.2	2.1	7.8

periment (Table I), indicates that the difference is not significant. On the other hand, the tabulated data indicate that the animals in group 3 clearly gained less than the animals of the other groups. It is possible in this instance that the additional trauma of 2 extra injections daily may be the cause of the decreased weight gain.

Side reactions. At no time did any of the treated guinea pigs display any evidence of local or systemic toxicity due to thonzylamine, either at the 12 or 18 mg daily dose.

Survival time. As in any long term experiment with guinea pigs, a small number of the experimental animals died from causes other than tuberculosis. These causes of death, usually bronchopneumonia or evident trauma, are easily differentiated from tuberculosis either at gross autopsy or during microscopic examination of the tissues. The few animals which clearly died from extraneous causes were routinely eliminated from further consideration. The percentages of surviving animals plotted against time indicated that the animals receiving the combined treatment (Group 3) survived the longest mean time, the streptomycin treated animals the next longest, the thonzylamine group the third longest, and the control groups the shortest. However, statistical analysis of survival time indicates (Table II) that the differences in survival times are significant only when the thonzylamine group is compared with the controls ($p = .02$) and when the streptomycin treated animals are compared with the controls ($p = .001$). The difference in survival times between those receiving the combined treatment and those receiving the streptomycin treatment is not significant statistically ($p = .1$).

Extent of tuberculosis. Considering only those animals in which tuberculosis was the cause of death, average gross autopsy scores were determined (Table III). The animals in group 4 were given streptomycin in doses too small to effect a cure, but in agreement with others(3), a distinct beneficial effect was obtained. The results were not improved by the addition of the antihistamine (Group 3). The animals receiving thonzylamine alone (Group 2) appeared, at autopsy, to exhibit less tuberculosis than the controls (Group 1). The difference is small, but in view of the random method of evaluation and the large numbers of animals, it is felt that the difference is real.

In the present experiment the thonzylamine decreased the extent of the disease, but not as significantly as it did in our earlier experiment(2). An explanation for the difference lies, we believe, in differences between the two sets of experiments. In the earlier experiment, animals were autopsied 3 months after infection, whereas, in the present experiment, death due to tuberculosis was taken as the end point. Since thonzylamine possesses no significant antimycobacterial activity, it can act only to diminish the allergic reaction of the host to tubercle bacilli. Once spread, however, the organisms would proliferate normally and would cause lesions. Thus, the effectiveness of the antihistamine would diminish as the disease progressed, and the difference in the extent of the disease in the control and the thonzylamine treated animals would tend to diminish with time. If this is so, one would expect a greater influence of the antihistamine drug to appear in the earlier short-range experiments than appears in the present long-range experiments.

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Microscopic examination. All animals of Groups 3 and 4 were examined, but in the larger groups, 25 sets of sections were selected at random from each group for examination. Study of Ziehl-Neelsen and hematoxylin-eosin stained sections disclosed no difference between the controls (Group 1) and thonzylamine treated animals (Group 2) with respect to type of lesions or to numbers of bacilli present. All animals in these two groups showed predominantly fibrotic lesions with moderate caseation which contained small numbers of acid fast bacilli. The streptomycin treated guinea pigs (Group 4), and those receiving both drugs (Group 3) showed more fibrosis, fewer caseating lesions, and bacilli were difficult to find. The lesions in the latter two groups were similar to the lesions described by Karlson and Feldman(3) in tuberculous guinea pigs given the same dose of streptomycin.

Discussion. Thonzylamine, an effective antihistaminic agent which is capable of suppressing allergic reactions, was used in this and in our preceding study in an effort to determine whether or not it would aid in the chemotherapy of infectious diseases. It is evident from the above experiment that thonzylamine causes a slight but statistically significant increase in the survival time of experimentally infected tuberculous guinea pigs, and this appears to be reflected in a lessened spread of the disease. With curative doses of streptomycin (unreported experiments) the disease responded promptly and no effect attributable to thonzylamine could be discerned. With sub-curative doses of the antibiotic, our findings were in agreement with those reported by Karlson and Feldman(3) but, here again, thonzylamine did not influence nor aid streptomycin therapy. It must be concluded that thonzylamine is not a practical aid in the therapy of experimental tuberculosis in guinea pigs, but the slight beneficial result still leaves open the possibility that antihistaminic drugs may be useful in combatting the allergic component of infectious diseases.

Conclusions. Thonzylamine caused a slight reduction in the extent of tuberculosis in experimentally infected guinea pigs and caused a slight but significant increase in the length of survival. It did not supplement the effects of streptomycin.

Received June 26, 1951. P.S.E.B.M., 1951, v77.

Evidence that Hyaluronidase is Not the Factor in Testicular Extract Causing Increased Vascular Permeability.* (18877)

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A factor capable of increasing vascular permeability was demonstrated to be present in bull testis extracts by Duran-Reynals(1). This finding has been amply confirmed(2-5). Duran-Reynals and others have assumed that hyaluronidase is responsible for both spreading and increase in vascular permeability.

We have investigated hyaluronidase preparations, more highly purified than those previously available, for vascular permeability-increasing activity. Two approaches were used: (1) a study of the local extravasation of an intravascular colloidal dye following

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* Aided in part by grants from the Chicago Heart Association and the Allen B. Wriseley Co. Food Research Fund.

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TABLE I. Influence of Purification of Testicular Hyaluronidase upon its Capacity to Increase Vascular Permeability in Rats.

	Hyaluronidase		Evidence of increased vascular permeability				
Exp No.	Preparation	Purity,† μ/mg N	Evans Blue conc, μg/ml blood corrected for body wt		Hemoglobin conc, g/100 ml		Visible edema and blueing
			Saline	Enzyme	Saline	Enzyme	
1	A	1,000	134 ± 2.7*	82 ± 5.3*	*	*	+
	C	55,000		116 ± 5.9			0
2	A	1,000			14.4 ± .37*	16.1 ± .15	+
	D	40,000				14.4 ± .15	0
	E	93,000				14.4 ± .35	0
3	B‡	600	134 ± 2.4	71 ± 2.2	12.4 ± .45	15.7 ± .70	+
	A	1,000		93 ± 5.6		14.7 ± .80	+
	F‡	36,000		129 ± 1.2		12.4 ± .99	0
	G	54,000		125 ± 2.1		12.2 ± .44	0
	E	93,000		122 ± 1.5		12.2 ± .55	0

* Values given are the means and their standard errors.

† Determined by turbidity-reducing method of Dorfman and Ott(8).

‡ These groups contained 4 animals, all other groups consisted of 5 rats each.

local injection of active material, and (2) a study of the appearance of edema, change in hemoglobin concentration and rate of disappearance of a colloidal dye from the blood following intravenous administration of enzyme preparations. Evidence is presented to show that purified hyaluronidase does not have vascular permeability-increasing activity.

Methods and materials. Female Sprague-Dawley rats weighing approximately 195 g were fed Purina chow and water *ad lib*. Testis hyaluronidase preparations were administered intravenously immediately following an injection of Evans blue, as previously described(5). Weights were taken at the time of injection. Thirty minutes after the administration of enzyme the concentration of the dye in the blood was determined and the animals were examined grossly for evidence of edema and dye in the 4 paws and snout. In some instances, hemoglobin concentration was measured.

Extracts of bull testis containing hyaluronidase activity† were fractionated with alcohol in the cold under controlled conditions of pH and ionic strength(7). Enzyme thus prepared varied in purity from 36,000 to 93,000 units per mg N. Approximately equivalent quantities of various preparations, in terms of hyaluronidase activity (2000 units), were administered and their effects on vascular permeability were compared with those of two crude preparations of enzyme; A, containing 1000 units per mg N and B, having 600 units per mg N. The latter preparation served as starting material for the more purified extracts (F, G, and E, see Table I). Hyaluronidase activity was determined by a turbidity reducing method, the unit being related to an arbitrary standard(8).

The effect of local injection of 2 extracts was tested in 5 rats. Following the intravenous administration of 2 mg of Evans blue, 0.05 ml of 0.85% saline containing 25, 2.5 and 0.25 units of preparation A was injected intracutaneously in the shaved skin of the back. Parallel injections of similar quantities of the purified enzyme, D, were made in the same animals. Five hundredths ml of 0.85% saline was injected intracutaneously in each rat as a control. Observations were made on the extravasation of dye around the injection sites 15 minutes following the administration of enzyme.

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Results. The data summarized in Table I demonstrate that each hyaluronidase preparation was administered in approximately equivalent quantities in terms of enzyme activity. They also demonstrate that the crudest enzyme preparations (600 and 1000 units per mg N), prepared essentially by the method of Claude and Duran-Reynals(9), had vascular permeability-increasing activity as manifested by the grossly apparent edema and tissue coloration, the increased hemoglobin concentration and/or dye disappearance. In sharp contrast, is the lack of such effects when enzyme preparations of substantially higher purity were tested (36,000 to 93,000 per mg N).

Local extravasation of dye in excess of the saline control was induced by the relatively crude testis extract, A, in quantities of 25 and 2.5 units, but not when 0.25 unit of hyaluronidase activity was administered. With the purified enzyme, D, only very slight evidence of increased dye extravasation was obtained when 25 units were administered locally. No increased loss of dye was seen with this preparation administered in smaller amounts.

Discussion. Relatively crude preparations of testicular hyaluronidase, prepared by modifications of the method of Claude and Duran-Reynals(9), have been used in the majority of previous investigations(1-5). It is not surprising, therefore, that similar effects on vascular permeability were obtained. Recently, Elster *et al.*(10) described testing a more highly purified preparation (35,000 units per mg N) in which vascular permeability-increasing activity was found. They used 3 to 4 times the amount of hyaluronidase we have used and noted that even with this quantity of enzyme the effect on vascular permeability was less prolonged than that produced by the cruder preparations suggesting that there may have been some reduction of the permeability-increasing activity in their material.

It has been reported that preparations having hyaluronidase activity showed no evidence of inducing increased vascular permeability. Zweifach and Chambers(11), using direct observation of mesenteric capillaries, did not observe permeability increases short of actual rupture of the capillary wall. Local injection of hyaluronidase-containing extracts into the skin of rabbits did not produce any leakage of intravascular dye in the hands of Rocha e Silva and Dragstedt(12). Both groups of investigators used preparations obtained from Dr. Karl Meyer at Columbia University. Danielli and Stock(13) also make mention of the fact that in their frog leg perfusion preparations they could find no evidence of vascular permeability increase but only of vasodilator activity. The source of their preparation is not mentioned.

The results reported in this paper indicate clearly that purification of the hyaluronic acid depolymerase present in testicular extract does not lead to purification of the factor capable of increasing vascular permeability. On the contrary, the vascular permeability-increasing activity disappears with purification of the enzyme. The capacity to increase vascular permeability resident in crude testicular extracts does not, therefore, appear to be due to its content of hyaluronidase.

Preliminary qualitative observations of the spreading of India ink and Evans blue in the skin of rabbits and rats indicate that purified hyaluronidase preparations retain the spreading effects despite loss of vascular permeability-increasing activity.

We have as yet little information on the chemical nature of the vascular permeability-increasing factor. The following facts are at hand and of some interest. It is extractable, at least in part, with 0.1 N acetic acid. At least a part of it is soluble in 0.3 saturated ammonium sulfate and precipitated by 0.7 saturated ammonium sulfate. It is retained by the dialysing membranes ordinarily in

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use. Heating at 70°C for 30 minutes destroys the activity. Work is in progress to purify and further characterize the substance in the testis extract capable of increasing vascular permeability.

Summary and conclusions. The intravenous or local administration of relatively crude bull testis extract is capable of causing increased vascular permeability in rats. Purification of the hyaluronidase component of this extract results in loss of its capacity to induce increased vascular permeability. The purified hyaluronidase appears to retain its spreading activity.

Received June 27, 1951. P.S.E.B.M., 1951, v77.

Failure of Testosterone to Alter the Responsiveness to Insulin in Diabetes Mellitus.[*] (18878)

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In previous studies(1,2) it was shown that the administration of desoxycorticosterone acetate to patients with diabetes mellitus altered the responsiveness to insulin. Patients who were initially insensitive were almost invariably made transiently more sensitive, whereas those who were initially sensitive became less sensitive or were unchanged. It was postulated(2) that the observed increase in sensitivity may have been due to the inhibition of contra-insulin factors from the anterior pituitary or adrenal glands. Cheng and Sayers(3) have postulated that the action of DCA in increasing sensitivity to insulin in the rat was due to the decrease in the output of 11-oxygenated adrenal steroids, secondary to the inhibition of ACTH release by the hypophysis.

The present studies are part of a series to evaluate the relative effectiveness of various steroids in altering insulin sensitivity in humans, and to determine if possible the chemical groupings associated with this phenomenon.

Procedure. Thirteen patients with well-controlled diabetes were studied. Insulin-sensitivity was determined by the Himsworth(4)-glucose-insulin tolerance test as previously described(2). After the determination of the initial sensitivity, testosterone propionate in doses indicated in Table I, was given intramuscularly for a period of 10 days, and the insulin sensitivity again determined.

Results. The initial and final values for the glucose-insulin areas are shown in Table I, and the glucose-insulin tolerance curves in Fig. 1. As has been previously described(2),

TABLE I. Glucose Insulin Areas Before and After Administration of Testosterone for 10 Days.

Daily dose of testosterone, mg	Patient	Glucose-insulin areas in mg min Before	After
10	W. M.	+ 7400	+6600
	E. T.	— 1800	— 924
	J. K.	— 140	+ 180
	J. A.	+ 1760	—1730
25	M. W.	+ 1240	—1900
	I. S.	+12,550	+5100
	E. R.	+ 3750	+4830
	W. K.	+ 2220	+2420
50	J. H.	+ 4300	+2650
	C. M.	— 650	+2340
	W. S.	+ 920	+1100
	G. P.	+ 1130	+2010
	W. L.	+ 900	+1310

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[*] Sponsored by the VA and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are a result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

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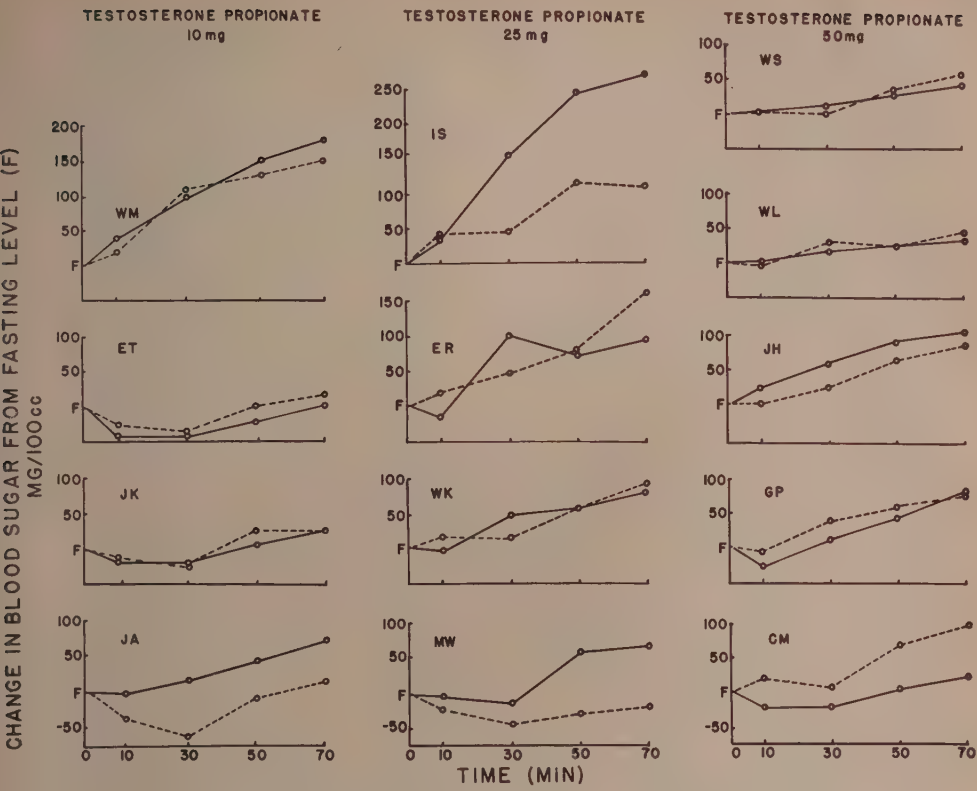

FIG. 1. Effect of continued administration of testosterone on the glucose-insulin tolerance test. Before, ————; after – – – – – –.

the degree of positivity of the glucose-insulin area indicates the degree of insensitivity to insulin. A distinct change in sensitivity was noted in only 1 patient (I.S.) whose daily dose of testosterone was 25 mg. A slight increase in apparent sensitivity was noted in 3 patients (J. A., M. W., and J. H.) while a slight decrease or no change in apparent sensitivity occurred in the remainder of the group. In no patient other than I. S., however, were the changes considered significant.

Discussion. The data indicate that in the doses used, testosterone was much less effective in altering responsiveness to insulin than was desoxycorticosterone. While the mechanism of the change in insulin sensitivity observed after the administration of DCA is not clear, the hypothesis that it occurred through the inhibition of hypophyseal or adrenal hormones still appears tenable. Testosterone has been shown to inhibit the pituitary output of gonadotropic hormones(5) and to depress the urinary excretion of corticoids(6), presumably through adrenal inhibition. The ineffectiveness of this steroid in altering responsiveness to insulin suggests, therefore, that either (1) the inhibition of the anterior pituitary may be selective with respect to the various pituitary hormones, (2) the change in insulin sensitivity may not depend on pituitary or adrenal inhibition, or (3) DCA may be more specifically effective than testosterone in inhibiting the output of contrainsular factors. As is shown in Fig. 2, the structure of desoxycorticosterone differs from that of testosterone only in that

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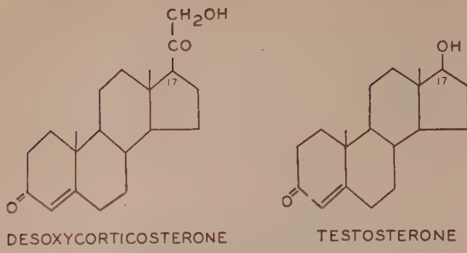

FIG. 2. Comparison of structures of desoxycorticosterone and testosterone.

the former has a side chain at C17, while the latter does not. It may be that the presence of this side chain is essential for the alteration of responsiveness to insulin.

Summary. The administration of testosterone propionate in doses of 10, 25, or 50 mg daily for a period of 10 days to 13 patients with diabetes mellitus failed to alter significantly the responsiveness to insulin in all but one. It is suggested that the presence of the side-chain attached to carbon atom 17 in the steroid molecule may be essential for this activity.

Received June 29, 1951. P.S.E.B.M., 1951, v77.

Nephrotoxic Action of dl-Ethionine.* (18879)

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Ethionine, a presumed antagonist of methionine, induces fatty changes in the liver(1), influences protein metabolism(2), and causes destructive changes in the pancreas(3,4). In the course of experiments in which enhancement of the pancreas-damaging effect of ethionine was found by the use of a protein depletion diet(5), renal changes were also observed. These are the subject of the present communication.

Experiments and results. Young male and female albino rats of the Wistar strain weighing from 125 to 250 g were used. One group of male and female rats was fed a complete Rockland rat diet. A second group was put for a period of 7 to 14 days on a protein depletion diet consisting of 77.6 parts corn starch, 15 parts hydrogenated cotton seed oil (Crisco), 4 parts salt mixture U.S.P. XIV, 4 parts ruffex, and 0.4 part Brewers yeast powder (Nutritional Biochemical Corp., Cleveland, Ohio). The yeast, according to the manufacturer, contains 50.3% protein. The methionine content based on 16% N averages around 1.8%. The protein depletion diet used contained therefore approximately 0.2% protein and 0.0036% methionine respectively. The animals received in addition one drop of cod liver oil twice weekly. All rats were given 1 mg dl-ethionine (α-amino-γ-ethylthyol butyric acid) (Nutritional Biochemical Corp.) per g body weight, divided in 3 doses, intraperitoneally. Animals on a complete diet were fasted for 16 hours prior to the administration of the drug. The possible protective effects of dl-methionine, glycine, and dl-alanine were tested by the administration of equimolecular amounts of these amino acids which were given intraperitoneally in 3 divided doses half an hour prior to the injections of ethionine to rats on the protein depletion diet. These rats were sacrificed 24 hours later.

Renal damage was seen in almost all of the

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* This work was supported by a grant from the Damon Runyon Memorial Fund for Cancer Research, Inc.

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FIG. 1. Sections of the kidney of a male rat on the protein depletion diet for 12 days and sacrificed 24 hr after ethionine administration. Necrosis of the distal portions of the proximal convoluted tubules in the inner cortical zone is widespread. Hematoxylin-eosin stain × 40.

animals on the protein depletion diet. Distinct changes in tubules located in the inner portion of the renal cortex were noticed as early as 6 hours following the administration of ethionine. There was marked condensation of the basal portion of the cytoplasm which stained intensely eosinophilic with the hematoxylin and eosin stain. The luminal portion of the cytoplasm was often fringed, hazy in outline and took the stain poorly. The nuclei were shrunken and pyknotic. After 13 hours some of the tubular cells had become frankly necrotic. In rats sacrificed 24 to 48 hours following the administration of ethionine destructive changes in the epithelium of tubules located in the inner zone of the renal cortex had become widespread in some of the kidneys (Fig. 1). The epithelium in damaged tubules was completely necrotic and the nuclei had disappeared entirely. Occasionally, however, pyknotic nuclei were still present in some of the damaged tubules. These changes resembled closely those seen following the administration of dl-serine(6). In animals that were allowed to live for 3 days evidence of early repair was found. Tubular epithelium started to regenerate and mitotic figures became numerous. Within the next day the necrotic tubular cells were partially replaced by flat cells which had a more basophilic staining cytoplasm. Due to the flatness of these cells the tubular lumen appeared dilated. In rats that lived for 7 days almost all of the destroyed tubular epithelium had been replaced but remnants of necrotic cells were still occasionally visible. Calcification was not seen. Casts were not noticed and the glomeruli remained intact. In an attempt to localize the sites of the renal damage, single nephrons were isolated and appropriately stained. The isolation of single renal tubules was performed by Dr. Jean Oliver. Necrosis was limited to the terminal portion of the proximal convoluted tubules. No other part of the nephron was involved. The extent of the renal involvement varied considerably. Its degree was expressed as 0, ±, 1+, 2+, and 3+. In kidneys considered to show ± damage only very few necrotic tubules could be observed on appropriate sections.

As can be seen from Table I, there is considerable difference between male and female rats and between animals on a complete or a protein depletion diet in the susceptibility of the renal cortex to the damaging action of ethionine. Male animals on both diets, showed more extensive involvement than female ones. On the complete diet, however, renal involvement was only light in the male and absent in the majority of the female rats. There was a marked increase in renal toxicity in rats on the protein depletion diet. A similar enhancement of the pancreas-damaging action in animals on a protein depletion diet has been described previously(5). Almost complete protection was observed in both male and female animals when methionine was given, while glycine and alanine were without significant influence.

Comment. Renal lesions were found on a diet low in protein with either carrots or turnips as the primary constituents(7,8). Since, however, rats on a diet containing 0.5%(8) or 1%(9) casein as protein source did not pre-

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TABLE I. Effect of Sex, Diet and Several Amino Acids on Renal Toxicity of DL-Ethionine.

Sex	Diet	Administered: Ethionine	Administered: Additional	Total No. rats	No. of rats showing lesions: 0	±	1+	2+	3+
Male	Complete	+	—	10	2	3	1	3	1
Female	"	+	—	12	8	2	0	2	0
Male	Protein depletion	—	—	7	7	0	0	0	0
"	" "	+	—	20	0	0	1	13	6
Female	" "	+	—	17	1	2	5	9	0
Male	" "	+	DL-methionine	8	6	2	0	0	0
"	" "	+	Glycine	6	0	0	1	3	2
"	" "	+	DL-alanine	6	0	0	1	3	1
Female	" "	+	DL-methionine	10	9	1	0	0	0
"	" "	+	Glycine	6	2	1	1	2	0
"	" "	+	DL-alanine	7	0	0	2	4	1

sent comparable renal changes, it was concluded that it was not the lack of protein but the persistent alkaline urine containing large amounts of calcium and phosphate which was responsible for the renal lesions(9). The depletion diet used in these experiments containing only 0.2% protein likewise did not induce renal damage. Protein depletion led, however, to marked increase in the susceptibility of the kidney to the nephrotoxic action of ethionine. In view of the fact that female rats on a complete diet very often failed to show any renal lesions the negative findings of Simpson, Farber, and Tarver(2) are thus easily explained.

Ethionine has been shown to inhibit to a marked degree the incorporation of radioactively labeled methionine and glycine into the proteins of the liver and kidneys of intact rats(2). These inhibitions as well as the fatty infiltration of the liver caused by ethionine are prevented by methionine but not by other amino acids including glycine and alanine(1). Methionine prevents also the pancreatic damage(3). The nephrotoxic action of ethionine is influenced in a similar manner by methionine but not by glycine or alanine. It is noteworthy that tissue repair in the kidney should set in 48 to 72 hours after the administration of ethionine, in spite of the fact that the animals remained on a diet that contained only traces of methionine.

Under different experimental conditions ethionine was found to exert an opposite effect. Mixed with the food it prevented the accumulation of liver fat and the hemorrhagic kidney degeneration on a low methionine, low choline diet(10). This is not necessarily contradictory to the present findings since much larger amounts of the drug reach the blood stream and kidneys when given by the parenteral route than when mixed with the diet. The beneficial effect of ethionine on the renal damage caused by choline deficiency probably involves a different biochemical mechanism.[†]

The greater susceptibility of male rats to the nephrotoxic action of ethionine is in agreement with previously established findings. dl-Serine(6,11) carbon tetrachloride(12), and chloroform(13) are more toxic to male than to female animals. The hemorrhagic necrosis in acute choline deficiency is likewise more pronounced in young male than female rats (14).

Summary. Ethionine induces necrosis of the distal portion of the proximal convolutions in the rat kidney. On a normal diet renal involvement is seen more frequently and to a greater extent in male animals. A pro-

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[†] No renal damage was found by Stekol and Weiss (*J. Biol. Chem.*, 1949, v179, 1048) in male rats given at the age of 24 to 35 days a complete diet containing 25% casein and 0.14 to .55% ethionine for a period of 27 days. Feeding of ethionine together with choline or methionine over a period of 3 months resulted in liver and kidney damage.

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tein depletion diet increases very markedly the susceptibility of the kidney to the nephrotoxic action of ethionine in male and female rats. Methionine inhibits the renal toxicity of ethionine while glycine and alanine are without effect.

The authors are indebted to Dr. Jean Oliver, professor of pathology, State University of New York, College of Medicine at New York City, for his cooperation and invaluable help.

Received June 29, 1951. P.S.E.B.M., 1951, v77.

Differences of Cortical/Medullary Ratio in the Adrenals of Normal Rats, Normal Rats treated with ACTH, and Renal Hypertensive Rats.* (18880)

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The existence of a direct relationship between heart weight and adrenal weight in male rats made hypertensive by the removal of one kidney and constriction of the other has been reported(1,2). It was shown that the relative degree of enlargement of the medulla exceeded that of the cortex, although by far the greater part of the weight increase of the whole gland was due to increase in size of the cortex(2).

The assumption that enlargement of the adrenals under conditions of stress is due to the effect of hypophyseal corticotrophin has been generally accepted(3). If increase in the size of the adrenals in severe or prolonged stress is due solely to the effect of hypophyseal corticotrophin, and if enlargement of the adrenals in renal hypertensive rats is an instance of the reaction to stress, then it would be expected that the administration of adequate doses of ACTH would bring about changes in the adrenals of normal rats similar to those found in renal hypertensive rats. The aim of the present report, in part, is to show that such is not the case. ACTH was given to normal rats and found to induce cortical but not medullary growth, within the limits of accuracy of the method. If any change in medullary size occurred it was very small. In contrast, the adrenal medulla of the renal hypertensive rat has been shown to increase to as much as 4 times the normal size. This is due to both cell hypertrophy and cell hyperplasia(2).

Experimental procedure. Four male rats of the Stanford colony were given 0.5 mg of ACTH (Armour) intramuscularly 4 times daily to a total dose of 25 mg each. They were then killed by exsanguination from the aorta under ether anesthesia and their adrenals weighed to the nearest milligram on a torsion balance. Seven normal control rats were killed similarly, as were 6 renal hypertensive males. Cortical/medullary volume ratios were determined by dividing each adrenal into 3 discs, embedding these in paraffin after fixation in Bouin's fluid and dehydration in dioxane, and filling several slides with sections. The slides were projected onto paper at 37 diameters magnification, drawn in outline, and the areas of cortex and medulla measured with a planimeter. A correction was made for vascular space in the medulla only. The individual cortical/medullary ratios given are based on a minimum of 100 sections through the adrenals and their statistical accuracy has been tested and verified by plotting the course of cumulative ratios(2).

Results. The data are given in Table I. The 32% increase of the ACTH treated rats over the normal rats in adrenal weight expressed as percent of a predicted value based on body weight, is statistically significant ($P < 0.02 > 0.01$). No significant change in medullary weight seems to have been produced

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* This investigation was supported by USPHS Grant No. H-811.

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TABLE I. Cortical/Medullary Ratios and Calculated Weights of Cortex and Medulla in Normal Male Rats, Renal Hypertensive Male Rats and Normal ACTH Treated Male Rats.

Body wt, g	Adrenals wt, mg	% normal*	Cortical/medullary ratio	Cortex, mg	Medulla, mg
		Normal male rats			
320	43	119	14.1/1	40.2	2.8
312	26	74	10.2/1	23.7	2.3
312	40	114	13.8/1	37.3	2.7
308	34	97	9.7/1	30.8	3.2
193	31	112	14.7/1	29.1	1.9
200	24	86	12.2/1	22.2	1.8
200	28	99	13.7/1	26.1	1.9
	Mean	100	12.6/1		
		ACTH treated rats			
303	48	139	15.4/1	45.1	2.9
275	43	130	13.5/1	40	3
170	37	140	13.1/1	34.4	2.6
175	32	121	16 /1	30.1	1.9
	Mean	132	14.5/1		
		Renal hypertensive male rats			
162	59	232	10.7/1	53.9	5.1
255	96	302	8.4/1	85.8	10.2
207	77	268	6.7/1	67	10
145†	55	229	10.1/1	50.1	4.9
246†	96	302	8.8/1	86.2	9.8
162†	62	248	8.1/1	55.2	6.8
	Mean	264	8.8		

* Predicted from body wt according to equations of Addis and Gray(5) based on data from this colony.

† On high protein (60% casein) diet. All other rats on stock diet containing 18% protein and .3% NaCl.

by the injections of ACTH. When the mean cortical/medullary ratio of the ACTH treated rats (14.7/1) is compared with that of the renal hypertensive rats (8.8/1) a highly significant difference ($P < 0.01$) is evident. Calculation of the weight of the medulla on the assumption that cortex and medulla are of equal density shows a great increase in medullary size in the renal hypertensive rats, which is due to cell hypertrophy and hyperplasia(2). Intracytoplasmic bodies were found in the cortical fasciculata in Bouin-fixed paraffin-embedded sections of the adrenals of all rats with chronic severe renal hypertension. These spherical droplets varied from 2 to 8 μ in diameter and often several were present in a single cortical cell. The nuclei of such cells usually showed nucleolar enlargement. The intracytoplasmic bodies stained brilliantly with leukofuchsin-sulfite after periodic acid oxidation. This positive PAS reaction could be blocked with the use of the technic described by McManus(4) consisting of treating the sections with acetic anhydride in pyridine, and restored by hydrolysis in dilute NaOH. The bodies were not digested by diastase. The solubility characteristics and the above histochemical reactions suggest that the material is a glycoprotein. At least the presence of a 1, 2 hydroxy grouping is to be presumed. Such bodies were very rarely present in the ACTH treated rats and not found at all in the controls.

Comment. This difference in the behavior of the adrenal in response to injections of ACTH and to the physiological situation of experimental renal hypertension can only mean that factors in addition to ACTH are concerned in the growth of the adrenal in the latter condition. The nature of these factors and the significance of the medullary enlargement is unknown. The hyperplastic, hypertrophic medulla is most probably producing an increased amount of hormones. It is possible, of course, that a dissociation of growth and hormone output has occurred, analogous to that seen in the thyroid after the administration of thiouracil. An explanation of this, however, would seem to be even more remote than one assigning to an increased production of medullary hormones some significance in the pathogenesis or maintenance of the elevated blood pressure in the renal hypertensive rat.

Summary. In the enlarged adrenals of renal hypertensive rats there is a relative increase in medullary size, whereas in the enlarged adrenals of rats treated with ACTH there is a relative decrease in medullary size. Large numbers of PAS positive intracytoplasmic bodies are found in the cortical fascicular zone in the adrenals of rats with severe chronic renal hypertension, but were not produced in comparable numbers with ACTH. It is con-

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cluded that factors in addition to ACTH are concerned in the growth of the adrenal in renal hypertension.

The author wishes to acknowledge the technical assistance of Mrs. Elizabeth Jensen.

Received June 29, 1951. P.S.E.B.M., 1951, v77.

Hepatic Artery Ligation in Rabbits Followed by Thoracic Caval Constriction.* (18881)

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Acute ligation of the hepatic artery, as shown by Markowitz(1), is compatible with life in dogs if an antibiotic such as penicillin is given in the postoperative period. This discovery has led to new approaches to the problem of ascites in animals and in man.

It has been indicated by Reinhoff(2), in cases of portal hypertension, that hepatic artery ligation might prevent the formation of ascites. Perfusion experiments have shown that blood flow through the hepatic artery in cirrhosis encounters less resistance than in the normal liver. The higher pressure of blood flow through the hepatic artery, with less resistance, tends to increase the resistance to the flow through the liver from the portal vein. The rationale of ligation of the hepatic artery in these cases was based upon the fact that lowering the intra-arterial pressure also lowers that in the portal vein. In a previously reported experiment(3), it was found that the ascites formed in rabbits by thoracic caval constriction was not affected by previous 2-stage portal ligation. The present experiment, as a continued study, was carried out to determine what effect hepatic artery ligation would have on the type of ascites produced by experimental constriction of the thoracic inferior vena cava. The fluid formed by such a procedure is believed to be the result of venous congestion in the liver. McKee(4,5) produced ascites in dogs within a few days by means of an adjustable aluminum band placed on the thoracic inferior vena cava midway between the heart and diaphragm. Volwiller(6) and Volwiller and co-workers(7), also working with dogs, used a cellophane band placed around the thoracic segment of the inferior vena cava. The fibrous reaction of the cellophane would cause a progressive constriction of the vessel lumen to approximately one-quarter of its original diameter within three to four weeks. This resulted in marked ascites.

Rabbits weighing 3 to 4 kg were used in this experiment. The diet consisted of stock rabbit pellets containing 17% protein, oats and water. All surgical procedures were executed under aseptic operating conditions. In the cases in which thoracotomy was performed, local anesthesia and positive endotracheal oxygen pressure were utilized. Postoperative intake and output were not measured.

Method and results. Ten control rabbits were subjected to hepatic artery division and ligation just distal to the gastroduodenal

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* Published with the permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or the conclusions drawn by the author.

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TABLE I. Comparison of Survival Rate in Rabbits With and Without Penicillin Following Hepatic Artery Ligation.

	No. Rabbits	Survived	Died	Avg days survived
Controls	8	0	8	2
Treated	18	12	6	5

branch. In every case, careful search was made for aberrant or extra hepatic branches of the hepatic artery. No antibiotics were given following surgery. On the 10 rabbits, 9 died within a period of 2 days, and 1 died after 5 days. Immediate post mortem examination revealed large amounts of gas present in the peritoneal cavity and gross necrosis of the liver. Culture of peritoneal exudate taken just before death and immediately afterward showed gas forming *Clostridium welchii* to be predominant. This is in accord with the experience of others (8-10) that the mechanism of death in liver autolysis is bacterial. It is thought that hepatic artery ligation allows activation of anaerobic bacilli in the liver by diminishing the oxygen supply to that organ. Table I gives results in this group.

In another group of 18 rabbits, the hepatic artery was ligated and divided in the same manner as in the control group. Postoperatively, however, 300,000 units of procaine penicillin were given twice daily for a period of 7 days. Twelve animals survived without visible effect. The 6 that died showed massive liver necrosis, gas in the peritoneal cavity, and presented a picture similar to that shown by the animals which received no penicillin. It was interesting to note that those animals that did not survive died in 4 to 6 days rather than in 2 days, as in the control group. (See Table I for comparison with the control group). The penicillin apparently contained anaerobic organisms for a time, at least. In none of the rabbits was ascitic fluid demonstrable in frequent paracentesis.

A second control series of 8 rabbits was operated upon. Each animal was subjected to constriction of the thoracic inferior vena cava midway between the heart and diaphragm, with a polyethylene band measuring .5 cm in width. The vein was measured carefully with calipers so that the vessel could be reduced as nearly as possible to 60% of its normal diameter. Closure of the chest was accomplished with interrupted silk, and the lungs re-expanded by means of positive endotracheal pressure. Following operation, the presence of ascites was determined by daily paracentesis. Ascites was found to occur in 3 to 4 days. Samples of fluid were removed every 7 days for a period of 2 months and analyzed for total protein and A/G ratio. The volume that could be removed at each sampling varied from 20 to 40 cc, with a specific gravity of 1.012 to 1.019. The values for total protein averaged 4.2 g per 100 cc, and the A/G ratio averaged 1.3. Table II outlines the 8 control experiments.

The 12 rabbits that survived hepatic artery ligation were reoperated upon again in 10

TABLE II. Ascitic Fluid in Rabbits with Thoracic Caval Constriction.

Exp.	Onset of ascites (days)	S. G.	T.P. (g/100 cc)	A/G
1	4	1.017	3.9	1
2	3	1.019	4.3	.9
3	4	1.012	4.5	1.5
4	4	1.018	4.6	1.3
5	3	1.012	3.9	1.7
6	3	1.014	3.8	1.6
7	4	1.018	4.3	1.4
8	4	1.016	4.2	1.4
		Avg	4.2	1.3

TABLE III. Hepatic Artery Ligation Followed by Thoracic Caval Constriction.

Exp.	Onset of ascites (days)	S. G.	T.P. (g/100 cc)	A/G
1	3	1.013	3.6	.9
2	4	1.016	3.8	1.4
3	4	1.014	4.4	2.1
4	3	1.014	4.3	1.2
5	4	1.016	4.2	1.7
6	3	1.017	4	1
7	3	1.015	3.6	1.3
8	3	1.017	4.3	1.4
9	2	1.016	3.8	1.5
10	3		4.3	1.6
		Avg	4	1.4

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days. The same technic of thoracotomy and thoracic inferior vena caval constriction, as employed in the second control group, was applied to this series. Positive endotracheal oxygen pressure was given throughout. No antibiotics were utilized in the postoperative period. Following operation, daily paracenteses were performed, and the formation of ascites was usually demonstrated in 3 to 4 days. This fluid was examined for total protein and A/G ratio every 7 days for a period of 2 months. No appreciable change in average total protein or A/G ratio was noted when compared with the fluid produced in the animals with thoracic inferior caval constriction alone. Total protein averaged 4.0 g per 100 cc, A/G ratio 1.4 and specific gravity 1.013 to 1.017. Table III outlines the results obtained in this group of animals.

Conclusion. 1. Acute hepatic artery ligation in the rabbit resulted in 100% mortality in this experiment. 2. Massive penicillin therapy following hepatic artery ligation in rabbits resulted in a 66% survival rate. 3. Ascites produced by thoracic caval constriction in the rabbit is not prevented or appreciably affected by previous hepatic artery ligation.

Received June 29, 1951. P.S.E.B.M., 1951, v77.

Influence of Vitamin B_{12} and Folacin on the Synthesis of Choline and Methionine by the Rat.* (18882)

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It was reported(1,2) that vit. B_{12} exerted a sparing action on the choline requirement of rats and chicks. It was later noted(3) that both folacin and vit. B_{12} were involved in the methylation process. Schaefer *et al.*(4) reported that chicks required vit. B_{12} for the synthesis of choline from methylaminoethanol and methionine or betaine. Jukes *et al.*(5) noted that vit. B_{12} was essential for the methylation of homocystine for growth in chicks fed a methionine-deficient diet. Oginsky(6) observed that livers of vit. B_{12}-deficient rats exhibit a lower ability to form methionine from homocystine and either betaine or choline as compared with those from animals dosed with vit. B_{12}. The role of vit. B_{12} and folacin in the methylation of guanidoacetic acid was reported by Dinning *et al.*(7). Stekol and Weiss(8) concluded that rats are able to grow on a diet free of all the known labile methyl group donors but

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* Published with the approval of the Director of the Alabama Agricultural Experiment Station. A preliminary report of part of this work was given at the annual meeting of the Fed. Am. Soc. for Exp. Biol., 1951, and at the 118th National Meeting, Am. Chem. Soc., Sept. 8, 1950. This investigation was supported in part by research grants from the American Cancer Society upon the recommendation of the Committee on Growth of the National Research Council, and from the National Cancer Institute of the National Institutes of Health, Public Health Service. Donations of crystalline vit. B_{12} and other vitamins were made by Merck and Co. and of Folacin (Pteroylglutamic acid) by Lederle Laboratories.

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TABLE I. Basal Diet.

		Vitamins added to supply mg/kg diet	
Alcohol extracted peanut meal*	25.00	Thiamine	2
Oxidized Casein†	6.00	Riboflavin	4
Sucrose	39.09	Pyridoxine	2
Vitamin Mix‡	5.00	Calcium pantothenate	10
Minerals (undried)§	4.40	Niacin	20
Cod liver oil‖	1.00	Inositol	200
Lard	19.00	Alpha-tocopherol	25
L-cystine	.26	Alpha-tocopherol acetate	25
DL-tryptophan	.10	2-Methyl-1,4-naphthoquinone	5
DL-homocystine	.15		

* After treating the peanut meal as described by Engel, R. W., *J. Nutrition,* 1948, v36, 739, it was extracted with methanol in a continuous Soxhlet apparatus for 7 days. Choline analysis on a 50-g sample was negative. Protein analysis = 60%; methionine supplied in the diet from 25% peanut meal = 0.11%.

† Prepared as described by Toennies, G., *J. Biol. Chem.,* 1942, v145, 667, as modified by Hove, E. L., Copeland, D. H., and Salmon, W. D., *J. Nutrition,* 1949, v39, 397.

‡ Thiamine, riboflavin, pyridoxine, calcium pantothenate, niacin and inositol added to sucrose to furnish the amounts listed per kg of diet.

§ Salmon, W. D., *J. Nutrition,* 1947, v33, 155.

‖ The tocopherol and 2-methyl-1,4-naphthoquinone were added to the cod liver oil.

TABLE II. Methionine Requirement of Rats Fed the Basal Diet Minus Homocystine Supplemented with 30 μg Vit. B_{12} and 2 mg Folacin per kg of Diet.

Dietary supplement— Choline, %	DL-methionine, %	No. of rats	Avg body wt gain, g	Incidence renal damage, %	Avg liver fat* (dry basis), %
.20	0	8	3	0	27
.20	.08	8	43	0	15
.20	.10	8	50	0	13
.20	.20	4	53	0	12
.20	.30	4	58	0	12
.20	0 †	8	58	0	13

* Total ether-extractable material.

† Methionine (0.18%) supplied by regular extracted casein replacing the oxidized casein.

which contained vit. B_{12}, folacin, and homocystine or homocysteine. The biological synthesis of labile methyl groups has been confirmed by Arnstein(9), du Vigneaud *et al.* (10), and Sakami(11). Sakami and Welch (12) reported that choline and methionine methyl groups are synthesized from formate. Arnstein(9) showed that methanol, formate, the β-carbon atom of L-serine and the α-carbon atom of glycine are precursors of choline methyl groups, but the incorporation of these precursors into the methyl group of choline appears to be slow as compared with their incorporation into the aminoethanol moiety.

In a continuation of studies on the interrelationship of vit. B_{12}, folacin, and choline, the effect of vit. B_{12} and/or folacin on the synthesis of both choline and methionine in the presence or absence of a dietary source of labile methyl was investigated. The *in vivo* synthesis of choline and methionine is implied by the favorable response observed in rats when the basal diet was supplemented with various precursors of these factors.

Experimental. Since a study of choline metabolism is directly related to the supply of dietary methionine, a diet deficient in both choline and methionine and supplemented with homocystine was employed. Composition of the basal diet is given in Table I. Weanling rats of the AES (Alabama Experiment Station) strain weighing 40-50 g were placed in individual cages and uniformly grouped with respect to number, weight, sex, and litter.

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10. du Vigneaud, V., Ressler, C., and Rachele, J. R., *Science,* 1950, v112, 267.
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TABLE III. Effect of Vit. B_{12} and Folacin on the Utilization of Choline or Betaine for Synthesis of Methionine from Homocystine.

Dietary supplement	Vit. B_{12}, μg/kg	Folacin, mg/kg	No. of rats	Avg body wt gain, g/2 wk	Incidence renal damage, %	Mortality, %
0	0	0	10	0	100	100
0	150	10	8	0	100	50
0.25% glycine, 0.20% DL-serine	150	10	8	−2	100	75
1% methanol	150	10	4	0	100	50
1% methanol, 0.05% aminoethanol	150	10	8	0	88	50
0.06% choline chloride	60	2	4	22	100	0
0.08% ” ”	60	2	8	35	0	0
” ” ”	60	0	8	28	50	0
” ” ”	0	2	12	17	50	0
” ” ”	0	0	8	14	75	0
0.10% ” ”	60	2	8	42	0	0
0.12% ” ”	60	2	8	49	0	0
0.16% ” ”	60	2	12	62	0	0
” ” ”	60	0	8	56	0	0
” ” ”	0	2	8	19	0	0
” ” ”	0	0	8	14	0	0
0.06% choline chloride, 0.06% betaine HCl	60	2	4	43	0	0
0.08% choline chloride, 0.04% betaine HCl	60	2	8	48	0	0
0.08% choline chloride, 0.08% betaine HCl	60	2	8	65	0	0

Feed and water were supplied *ad lib*. All rats were necropsied after death or at the end of a 14-day experimental period and the kidneys were carefully examined for gross pathological lesions. In general, each treatment consisting of 4 rats per group was repeated 1 to 3 times.

The additional dietary methionine supplement required for maximum growth was approximately 0.10% when the basal diet minus homocystine, was supplemented with adequate choline, vit. B_{12}, and folacin (Table II). At the 0.30% level of DL-methionine, growth was equal to that obtained when alcohol-extracted casein was used in place of the oxidized casein. Dietary supplementation of 0.08% DL-methionine supported nearly maximum growth (43 g per 2 wks). When the diet was supplemented with 0.20% choline chloride, the kidneys were protected against hemorrhage even though the diet was deficient in methionine; however, liver fat was increased from 12% to 27% (dry basis) by the omission of the methionine supplement.

The requirement for dietary choline to supply choline *per se* and sufficient methyl for methionine synthesis from homocystine is indicated by the data in Table III. Supplementation of the homocystine basal diet with vit. B_{12} and folacin and either glycine and serine, or methanol and aminoethanol was ineffective in preventing renal hemorrhage and for the support of growth of the weanling rats. The effect of either vit. B_{12} or folacin, or both folacin and vit. B_{12} on the utilization of choline for the methylation of homocystine was studied at 2 levels of choline, 0.08% and 0.16%, which supported suboptimal to optimal growth, respectively. (Table III). When the diet was supplemented with 0.08% choline chloride, both folacin and vit. B_{12} were essential for protection against renal damage and for support of fair growth (35 g in 2 weeks). Increasing the choline chloride level to 0.16% without vitamin B_{12} and folacin protected against renal damage; however, average weight gain was only 14 g in 2 weeks. The addition of vit. B_{12} alone to the diet supported maximum growth (56 g in 2 wks), whereas the addition of folacin alone was without benefit.

As a methyl donor for the synthesis of methionine from homocystine, betaine was as effective as choline, mole per mole (Table III), in the presence of vit. B_{12} and folacin. When the diet containing 0.08% choline chloride was supplemented with 0.04% betaine

TABLE IV. Role of Vit. B_{12} and Folacin on Synthesis of Choline and Methionine from Betaine, Aminoethanol and Homocystine.

Dietary supplement						
Betaine HCl, %	Amino-ethanol, %	Vit. B_{12}, $\mu g/kg$	Folacin, mg/kg	No. of rats	Avg body wt gain, g/2 wk	Incidence renal damage, %
.32	0	0	0	4	1	100
.32	0	60	2	8	25	62
.32	.03	0	0	4	−1	100
.32	.03	60	0	8	0	100
.32	.03	0	2	4	0	100
.32	.03	60	2	8	40	0
.35	0	0	0	4	8	100
.35	0	60	2	8	32	25
.35	.04	0	0	8	6	100
.35	.04	60	0	4	31	50
.35	.04	0	2	4	3	100
.35	.04	60	2	8	42	0
.41	0	0	0	4	9	100
.41	0	60	2	4	34	25
.41	.05	0	0	4	8	100
.41	.05	60	0	4	32	0
.41	.05	0	2	4	15	50
.41	.05	60	2	4	45	0
.60	0	0	0	4	26	25
.60	0	60	0	4	39	0
.60	0	60	2	4	43	0
.60	.06	0	0	4	33	0
.60	.06	60	0	4	42	0
.60	.06	0	2	4	30	0
.60	.06	60	2	4	43	0

HCl, growth was equal to that obtained with 0.12% choline chloride. Likewise, 0.08% choline and 0.08% betaine were equally as effective as 0.16% choline.

By using varying levels of betaine to replace an equivalent amount of choline, it was noted that for the replacement of metabolic choline, *per se*, 3 moles of betaine were required to replace 1 mole of choline. When the basal diet was supplemented with vit. B_{12} and folacin, 0.10% to 0.12% choline chloride (Table III) was as effective as 0.32% to 0.41% betaine HCl plus aminoethanol (Table IV) for promotion of growth and protection against renal damage. The synthesis of choline and methionine from betaine, aminoethanol and homocystine is shown by the data in Table IV. Supplementing the basal diet with betaine or betaine and aminoethanol failed to support growth and prevent renal hemorrhage. The addition of vit. B_{12}, folacin and 0.32% or 0.41% betaine resulted in an incidence of renal damage of 62 or 25% respectively. The further addition of aminoethanol gave complete kidney protection and supported near maximum growth. Rats fed the basal diet supplemented with 0.32% betaine HCl and aminoethanol required both vit. B_{12} and folacin for normal development. By increasing the level of betaine to 0.41%, vit. B_{12} (without folacin) protected against renal damage and supported fair growth. As the betaine level was increased to 0.60%, aminoethanol was not required for the prevention of renal damage (Table IV). The addition of vit. B_{12} alone to the diet was as effective in promoting growth as were both vit. B_{12} and folacin.

Discussion. The earlier studies(3), in which it was reported that vit. B_{12} and folacin exerted a sparing action on the choline requirement of rats, can now be interpreted as being the result of the function of these vitamins in the biological synthesis of choline and methionine. By using a diet deficient in choline and methionine, the importance of vit. B_{12} and folacin in the synthesis of choline and methylation of homocystine was demonstrated.

Vit. B_{12} was as effective as a combination of vit. B_{12} and folacin for the support of maximum body weight gain when the diet contained an ample methyl supply (0.16% choline). When the methyl supply was suboptimal (0.08% choline) both vit. B_{12} and folacin were essential. A possible explanation of these data and recent observations(8,12) is that folacin functions primarily in methyl synthesis and vit. B_{12} functions in either the transfer or utilization of labile methyl.

For the methylation of homocystine to methionine, betaine is at least as effective a methyl donor as choline. When animals are fed a diet deficient in choline, 3 moles of betaine are required to replace 1 mole of choline. This substantiates previous reports (13,14) that betaine replaces choline *per se* mainly by virtue of supplying one methyl group per mole of betaine.

Vit. B_{12} and folacin were essential for the synthesis of choline and methionine from aminoethanol, homocystine, and a limited supply of betaine. Under the experimental conditions employed, the amount of biological synthesis of labile methyl or of the utilization of methyl from methanol, or synthesis of the entire choline moiety, was insufficient to meet the entire choline requirement necessary for the prevention of renal hemorrhage in the *weanling rat*.

Summary. The role of vit. B_{12} and folacin in the biosynthesis of choline and methionine has been studied and the following conclusions are warranted: 1. In the presence of adequate methyls (from choline), vit. B_{12} alone was as effective for methionine synthesis as when combined with folacin. When fed alone, folacin was ineffective. 2. In the presence of a limited supply of methyl groups, both folacin and vit. B_{12} were essential for the normal development of weanling rats. 3. Vit. B_{12} and folacin were essential for the synthesis of choline and of methionine from aminoethanol, homocystine, and a limited supply of betaine. 4. As a methyl donor for the synthesis of methionine from homocystine, betaine was at least as effective as choline, mole per mole. 5. Under the experimental conditions employed, the rate of synthesis of the methyl group or utilization of methanol for the methylation of homocystine to methionine and aminoethanol to choline was not fast enough to support normal development of *weanling rats*.

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Received July 2, 1951. P.S.E.B.M., 1951, v77.

Changes in Acetylcholine Content of the Brain During Exposure to Cold. (18883)

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That acetylcholine (ACh) may play a role in the temperature regulatory mechanism of the mammalian organism and that its effect might be modified by changes in body temperature are suggested by the findings of several investigators. Thus, White(1) in 1929 reported that atropine (an anticholinergic drug) in moderate or excessive amounts produced a marked hyperthermia in infants and children. On the contrary, Burn and Dutta(2) showed that the body temperature of mice is lowered after atropine administration. Grosse-Brockhoff(3) reported therapeutic effects of atropine in hypothermia by diminishing excessive vagal influences and increasing the vascular tonus. Tournade and Curtillet (4) found the lethal dose of ACh to be considerably reduced in cold animals. It seems likely that the cardiovascular system is primarily involved in this change. Noell(5) has shown, however, that the brain also undergoes changes. He observed certain alterations in the electrical response of the cerebral cortex in rabbits whose temperature had been depressed to 28° to 34°C by submersion, which were similar to those obtained on uncooled rabbits receiving low concentrations of eserine intravenously. These experiments suggested that an investigation of the ACh content of tissues might show an increase in animals exposed to cold. The following study reports the results of such measurements on the brain.

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* Maj., USAF.

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2. Burn, J. H., and Dutta, N. K., *Nature,* 1948, v161, 18.

3. Grosse-Brockhoff, F., German Aviation Medicine in World War II, U. S. Govt. Print. Office, Wash., D. C., v2, ch. VIII-E, 1950.

Experimental. Free and total (free plus bound) ACh concentrations in rat brain were determined by the leech method described by MacIntosh and Perry(6). Rats were decapitated immediately on removal from the cage and the brain tissue anterior to the anterior quadrigemina removed, weighed, and homogenized in eserine-Locke's solution.[‡] A Potter-Elvehjem homogenizer was employed. The maximum elapsed time between decapitation and immersion of the excised brain was 30 seconds. We found the leech muscle extremely sensitive to hydrogen ion concentration, particularly with respect to relaxation between test contractions. Since satisfactory relaxations were observed at pH 7.0, all solutions were adjusted to within 0.05 pH[§] unit of this value before immersion of the leech muscle. No muscle was used unless it contracted at least 7 cm (as read at the end of a lever arm 20 cm from the fulcrum) against a force of 8.5 g cm, when exposed for 3 minutes to acetylcholine concentration of 0.1 μg per ml. The homogenates were diluted to produce contractions equivalent to those observed with ACh standards of 0.03 to 0.05 μg per ml. A blank was determined on each sample by destroying the ACh with alkali and boiling prior to analysis.

TABLE I. Brain ACh Concentration of Control and Chilled Rats (−12°C for 2 Hr).

Group	No. rats	ACh (μg/g wet wt) Free	SE$_M$*	Total	SE$_M$*
Control	29	1.15	.07	1.99	.11
Cold	30	1.54	.06	2.45	.18
P		<.01		.04	

* Standard error of mean $= \pm \frac{\sigma}{\sqrt{N}}$.

The accuracy of the assay was determined by recovery studies using the brains of 15 male rats selected without regard to weight, age, or strain. The brains were analyzed as described above. To an aliquot portion of the analyzed homogenate, known quantities of ACh were added and the recoveries calculated. Recoveries averaged 99 ± 11%.

Male, Sprague-Dawley rats of the same age (6 months) and weight (250 to 300 g) were selected to determine changes induced by acute cold exposure. Half the animals were exposed to a temperature of −12°C for 2 hours, at which time their brain ACh was determined individually, as already described. The other half remained at room temperature (25°C), but were otherwise treated identically. None of the animals died from the cold, but the average body temperature had fallen to 15°C at the end of the exposure period. A significant increase in both free and total ACh of the cold animals is shown in Table I.

Discussion. A possible explanation for the observed increase in ACh after cold exposure is the reduced activity of the rat under these conditions. An inverse relationship has been shown to exist between brain ACh and activity. Thus Richter and Crossland(7) reported a rise of 40% above normal levels during anesthesia, and an even greater increase in deep narcosis has been claimed by other

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4. Tournade, A., and Curtillet, E., *Compt. Rend. Soc. de Biol.*, 1936, v121, 505.

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6. MacIntosh, F. C., and Perry, W. L. M., Methods in Medical Research, Year Book Pub. Co., Chicago, 1950.

[‡] NaCl, 6.43 g; glucose, 0.715 g; NaHCO$_3$, 0.36 g; KCl, 0.30 g; CaCl$_2$, 0.07 g. Dilute to 1 liter and add 0.42 mg eserine sulfate.

[§] Determined with Cambridge pH Meter, Model R.

7. Richter, D., and Crossland, I., *Am. J. Physiol.*, 1949, v159, 247.

workers(8). Another possibility to be considered is a change in the relative rates of synthesis and hydrolysis of ACh produced by a lowered body temperature. Further studies are necessary to decide whether these or other alterations are responsible for the ACh increase.

Summary. Exposure to –12°C for 2 hours significantly increased both the free and total acetylcholine concentration of rat brain.

The statistical help given by Dr. William F. Taylor, Department of Biometrics, is acknowledged.

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Received July 2, 1951. P.S.E.B.M., 1951, v77.

Isolation from Human Sera in Egypt of a Virus Apparently Identical to West Nile Virus.* (18884)

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This report describes the isolation from human sera collected in Egypt, of 3 strains of a virus characterized by the capacity to produce encephalomyelitis in monkeys, mice and hamsters, and immunologically related to West Nile virus. The circumstances under which these virus strains were isolated were as follows. In the course of an "antibody survey," carried out in July 1950, one of us (J.R.P.), collected serum from 251 Egyptians living within a Rural Health District known as Markaz Callub, established by the Ministry of Public Health with the cooperation of the Rockefeller Foundation, and located some 30 km. north of Cairo.‡ Most of the Egyptians were children and most of them were attending a Dispensary. Apparently at least 3 of the children happened to be in stages of viremia at the time they were bled, for it was from their sera that the strains were isolated.

All 3 strains were subsequently found to be antigenically related to each other and to the West Nile virus. The latter virus was isolated at the Yellow Fever Research Institute in Entebbe from a human serum obtained in 1937 in Uganda, about 2000 miles south of Cairo(1), but it has not been recovered in nature since then. However, serological evidence obtained by Smithburn(2) pointed to widespread human infection with this virus in Central Africa, with 1 to 46% of the various population groups studied there yielding positive tests for neutralizing antibodies to West Nile virus. In like manner evidence has been obtained in the present study to show that antibodies to the newly isolated Egyptian virus are present in a very large percentage of normal individuals living in the vicinity of Cairo.

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* Laboratory work carried out in New Haven under the auspices of the Commission on Virus and Rickettsial Diseases, Armed Forces Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D. C.

† Fellow of the Israeli Government.

‡ Preliminary arrangements for carrying out this investigation and survey were made with the National Foundation for Infantile Paralysis, and with the U. S. Naval Medical Research Unit (NAMRU) No. 3 at Cairo, Egypt. We are indebted to Capt. J. J. Sapero, MC, U. S. N., Commanding Officer of NAMRU No. 3, for providing laboratory facilities and for the necessary clearances obtained from the Under Secretary of State for Medical Affairs in the Egyptian Government.

We are also indebted to Dr. W. A. McIntosh and Dr. J. M. Weir of the International Health Division of the Rockefeller Foundation at Cairo, for indicating sites for the collection of clinical material and data; and for providing professional and technical help, and other facilities.

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Isolation of virus. Each of the 251 Egyptian sera referred to above were tested for the presence of poliomyelitis antibodies by the addition of Lansing poliomyelitis virus and subsequent inoculation of the serum-virus mixture into the brain of adult Swiss mice. During the course of 3 of these 251 tests, (on sera Nos. 19, 21 and 101), the inoculated mice (8 used for each test) developed signs of encephalitis rather than the myelitis typical of the Lansing virus. From these mice 3 strains of a virus were recovered which did not prove to be Lansing virus. Re-examination of each of the 3 sera alone yielded viruses capable of producing encephalomyelitis in mice. In one instance the serum had an intracerebral titer of $10^{-2.5}$ in mice.

The virus in one serum was found to grow directly in the chick embryo, and a line of the virus has been maintained in embryonated eggs without its ever having been in mice. Infection in the egg may be recognized by the death of the embryo and by passage of embryo suspensions to mice in which the typical encephalitic disease is produced.

The agent readily passed bacterial filters (Seitz EK, Corning UF glass fritted).

Prior to and at the time these strains were isolated, there was no West Nile virus in our laboratory nor have we ever had a strain of this virus in our laboratory before.

Host range of Egyptian virus. Species susceptible to infection by the Egyptian virus include the mouse, hamster, monkey, chimpanzee, and chick embryo. Disease has not been produced in cotton rats, guinea pigs, and rabbits; however these latter species produce antibodies in high titer following a single intracerebral inoculation of virus. Rabbits also failed to respond to the application of virus to the scarified cornea.

Adult mice are more susceptible to the virus following brain inoculation, the titer of infected mouse brain by the intracerebral route being $10^{-7.5}$ and by the intraperitoneal route, about 10^{-3} and irregular. On the other hand newborn mice are equally susceptible to both intracerebral and intraperitoneal routes of inoculation, the titer by either route usually being $10^{-7.5}$. The titer in chick embryos is over 10^{-4} by either dropping the virus on the chorio-allantoic membrane or inoculating it into the yolk sac.

Rhesus monkeys are very susceptible to intracerebral (*i.c.*) inoculation of the virus: cynomolgus monkeys perhaps less so. The disease in monkeys (resulting from *i.c.* inoculation) is characterized by viremia, fever, ataxia, and prostration. Some monkeys may be left with a severe flaccid paralysis of the extremities resembling poliomyelitis, others show marked improvement with no signs of the disease after a period of one or 2 months. Histologically the intracerebrally inoculated rhesus monkeys have an extensive encephalomyelitis involving the cerebellum as well as other areas of the brain and cord. Following intraperitoneal and intramuscular inoculation, the picture is different, some monkeys exhibit a temporary viremia without fever about 3 days after injection, while none show signs of encephalomyelitis, nor has it been evident histologically in sacrificed monkeys.

Chimpanzees develop a silent infection without fever, following intracutaneous inoculation of virus (Fig. 1). Following a period of viremia which lasts for 3 days, neutralizing antibodies begin to appear, and are present in significant titer by the 6th day reaching their maximum by the 2nd to 3rd week. Complement fixing antibodies make their appearance a few days after neutralizing antibodies are detectable. Virus does not appear in the throat or feces during the 2 week period following its inoculation. The response of these animals to feeding of virus is unlike that following ingestion of poliomyelitis and Coxsackie (C) viruses by these animals. Again Egyptian virus cannot be detected in the throat or stools, and antibodies fail to appear.

Immunological reactions. The 3 strains of the Egypt virus are related to each other by cross neutralization and cross complement fixation tests. The virus is neutralized by and fixes complement in the presence of West Nile immune serum. It is neutralized in an irregular fashion and to a lesser degree by certain Japanese B and St. Louis immune sera, in similar fashion to West Nile virus

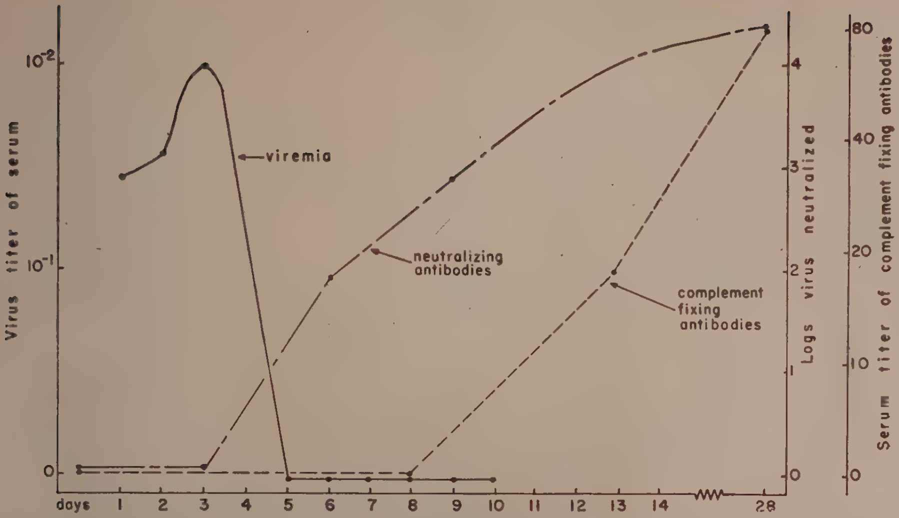

FIG. 1. Infection of 4 chimpanzees with the Egyptian virus. Each point represents the average of 4 determinations, each made on a different chimpanzee.

(3-5), while some Japanese B and St. Louis sera fail to neutralize the virus. Neutralization tests with the Egyptian virus may be carried out in adult mice (intracerebral route) or in newborn mice (intraperitoneal route). In many tests no difference has been observed in the sensitivity of the test carried out in either animal; however as with West Nile virus(6), in some tests the neutralization index of a serum has been 1 to 1.5 logs greater when infant mice were used.

The virus is not neutralized by sera containing antibodies to the following viruses:[§]

Dengue
Eastern equine encephalomyelitis (EEE)
Western equine encephalomyelitis (WEE)
Louping ill
Russian spring-summer encephalitis
Venezuelan encephalitis
Rift valley fever
Lymphocytic choriomeningitis (LCM)
Encephalomyocarditis (EMC, Mengo, Col.-SK, MM)
Vaccinia
Semliki
Bunyamwera
Uganda S
Zika
Ntaya
Bwamba
Mengo (EMC)
Coxsackie, Type Conn.—5
” ” Texas—1
” ” Ohio—1
Poliomyelitis, Type Lansing
” ” Brunhilde
Mouse encephalomyelitis, Theiler's FA
” ” ” GD7

In addition the virus was not neutralized by 4 pools of human gamma globulin prepared in the U. S. in 1945, 1949, and 1951.

Sera obtained from monkeys and chimpanzees following infection with the Egyptian virus have been found to react with West Nile virus in both neutralization and complement fixation tests.[‖] In addition certain Egypt virus immune sera have been found by Dr. I. Ruchman to neutralize Japanese B encephalitis, epidemic Keratoconjunctivitis, and St. Louis encephalitis viruses, but these crossings have been less pronounced than the reaction with homologous virus. In the complement fixation test, the

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[§] We are particularly indebted to Dr. Joel Warren of the Army Medical Center, to Dr. Kenneth C. Smithburn of the Rockefeller Foundation, to Dr. Albert B. Sabin of the Children's Hospital Research Foundation, Cincinnati, Ohio for some of the sera used in these tests.

TABLE I. Antigenic Relationship of Egyptian Strains by Complement Fixation.

Antigen	Dilution of serum*									
	Mouse. Egypt–19	Monkey 5166. Egypt–19	Monkey 5160. Egypt–101	Monkey 5433. West Nile	Mouse. St. Louis	Mouse. Jap B.	Monkey. LCM	Guinea pig. EEE	Monkey. Dengue	Monkey. Normal
EV-19	64	128	256	32	4	16	0	0	0	0
EV-21	64	256	512	256	32	16	0	0	0	0
EV-101	>64	256	512	256	<8		0		0	0
West Nile	64	128	512	256	32	32	0	0	0	0
St. Louis	16		4	0	256	8	0	0	0	0
Jap B.	32		16	0	32	64	0	0	0	0
L.C.M.	0		0	0	0	0	64	0	0	0
EEE	0		0	0	0	0	0	>256	0	0
WEE	0		0	0	0	0	0	0	0	0
Normal	0		0	0	0	0	0	0	0	0

* These tests were carried out by a modified Kolmer technic as used in this laboratory(7). Species is listed in which serum was prepared for indicated virus. Many of the antigens used and some of the sera were kindly furnished by Dr. Herald R. Cox, Lederle Laboratories, Pearl River, N. Y.

TABLE II. Clinical Record of 3 Egyptian Children with Viremia.

No.	Initials	Sex	Age in years	Diagnosis
19	S.A.F.	M	1	Impetigo
21	N.A.I.	F	10	? Malaria
101	M.A.	M	2	? Bronchitis

Egyptian strains cross react strongly with the West Nile virus and to a lesser degree with Japanese B and St. Louis encephalitis viruses. They fail to show any cross reactions with the viruses of western and eastern equine encephalomyelitis, lymphocytic choriomeningitis, and dengue (Table I).

Type of "Patient". The great majority of children from whom these sera were collected were thought to be normal at the time, although some had minor ailments, such as skin diseases, mild respiratory infection, or diarrhea. The Dispensary diagnoses in the 3 children with viremia are listed in Table II. There was no evidence that any of these 3 children was severely ill at the time blood was taken, but from the Dispensary diagnoses, it is likely that fever may have been present in N.A.I. (No. 21).

Local serological evidence of infection. Following the isolation of this virus from 3 of the 251 sera collected in Egypt, a search was made for neutralizing and complement fixing antibodies to the virus in the other local sera. Neutralization tests were carried out on 228 sera using undiluted serum and 2 to 2.5 logs of the No. 101 strain of the Egyptian virus, and at least two-thirds of the inoculated mice had to survive for a serum to be called positive. The complement fixation tests were carried out on 215 sera, using the No. 19 strain as antigen and reading the end point at 50% hemolysis. A positive serum was one in which at least 3 fifty per cent units of complement were fixed when the serum had been diluted 1:4. As shown in Fig. 2, both neutralizing and complement fixing (*c.f.*) antibodies were found in this human population to a marked degree. Both curves are similar except for the infants under 6 months who were found to contain neutralizing but not *c.f.* antibody. It appears that infection with this agent was widespread in this population in 1950. Maternal antibodies disappeared by the age of

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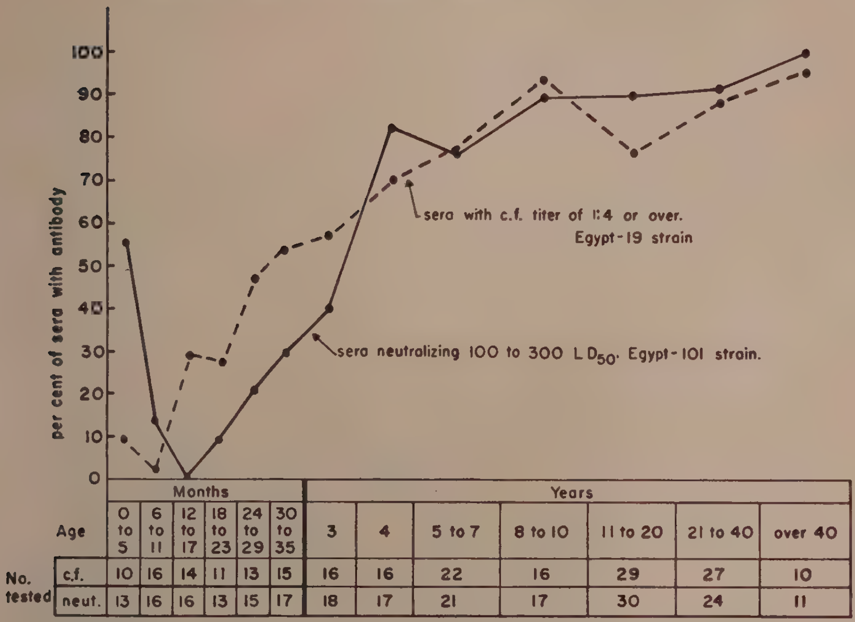
‖ We wish to acknowledge the generous cooperation in this work of Drs. Kenneth Smithburn, New York, Joel Warren, Washington, and Isaac Ruchman, Cincinnati, who have also found that our Egyptian immune sera neutralize the strain of West Nile virus used in their respective laboratories. Using the complement fixation test, Dr. J. A. Kerr, New York, also found that the sera of 2 monkeys convalescent from Egypt virus infection reacted with the West Nile virus used in his laboratory.

7. Kraft, L. M., and Melnick, J. L., *J. Exp. Med.*, 1950, v92, 483.

** These tests were largely carried out by one of us (N.G.).



CAIRO SERA, July 1950

Complement Fixing and Neutralizing Antibodies to Egyptian Strains of West Nile Virus

		Months						Years						
Age		0 to 5	6 to 11	12 to 17	18 to 23	24 to 29	30 to 35	3	4	5 to 7	8 to 10	11 to 20	21 to 40	over 40
No. tested	c.f.	10	16	14	11	13	15	16	16	22	16	29	27	10
	neut.	13	16	16	13	15	17	18	17	21	17	30	24	11

FIG. 2. Distribution of neutralizing and complement fixing antibodies in the normal population in the vicinity of Cairo at the time the virus-containing sera were obtained.

6 months, and then the children began to build up their own antibodies quite rapidly so that at the age of 4, 70 to 80% of the group had both neutralizing and *c.f.* antibodies. Both antibodies were maintained in the population through the age of 40 and above.

The occurrence of a viremia both in man and in experimental hosts and the failure to detect virus in the throat or stools of infected primates lead us to suspect that a biting insect acts as a vector in the transmission of the infectious agent. Obviously further work on sera collected in subsequent years from this area will be required to settle the many questions which have been raised by this study.

Summary. 1. Three strains of a filterable virus closely related to West Nile have been isolated from the blood of children living on the outskirts of Cairo, Egypt. 2. The 3 strains of virus appear to be immunologically identical. 3. In addition to a strong relationship and apparent identity with West Nile virus, the Egyptian virus crosses, although to a lesser degree, with both Japanese B and St. Louis encephalitis viruses. This was found by both neutralization and complement fixation tests. 4. The virus produces a marked encephalomyelitis in monkeys inoculated intracerebrally. Lesions are found throughout the spinal cord and brain including the cerebellum. 5. Chimpanzees develop a silent infection following intracutaneous infection of the virus. This is characterized by a viremia lasting 3 days followed by the appearance of both neutralizing and complement fixing antibodies. 6. The Egyptian virus was not neutralized by human gamma globulin collected in the United States, nor by 24 different hyperimmune sera, each containing antibodies to an antigenically distinct virus type. 7. Infection with this virus has been widespread in the local Egyptian population in 1950, with more than 70% of the inhabitants aged 4 years and over having both neutralizing and complement fixing antibodies.

Received July 3, 1951. P.S.E.B.M., 1951, v77.

In Vitro Effect of Testosterone on Respiration of Mouse Liver and Mammary Tumor Slices. (18885)

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The known metabolic effects of androgens (1) suggested that studies on the *in vitro* effects of testosterone on the respiration of tissue slices would be of value in elucidating the above phenomena. In this report a comparison between the liver and spontaneous mammary carcinoma of the mouse is presented.

Method. The studies were carried out by the Warburg method with the modified flask and procedure of Marsh(2). The testosterone‡ was dissolved in 0.2 ml of 95% ethyl alcohol to which was added 12 ml of the Ringer's phosphate medium(2). The medium for the control flasks was prepared in an identical manner but without the testosterone.

The liver and spontaneous mammary carcinoma were obtained from the same female mouse¶ of either the C_3H or dba strain. The tissues were immediately removed after killing the animal and placed into separate beakers containing Ringer's solution buffered with N/150 phosphate mixture of pH 7.4(2) chilled in an ice-water bath. The tissue slices were prepared immediately and the oxygen consumption was determined usually in duplicate occasionally in triplicate. Measurements were made at 37.5° in air for 1.5 hour at intervals of 30 minutes. Oxygen consumption was calculated in mm^3 per hour per milligram of dry weight of tissue (Qo_2). Duplicates which differed more than 5% were discarded.

Results. The presence of testosterone in the fluid medium produced identical results on the oxygen consumption of liver and spontaneous mammary tumor slices (Fig. 1). At a concentration of 0.035 micromolar, testosterone produced a small increase in the respiration of the tissues. As the amount of testosterone was increased, the enhancing effect disappeared and a progressive inhibitory action occurred.

Discussion. The pattern of response obtained in the adult tumor bearing female mouse is similar to that reported(3,4) for the liver and diaphragm of growing young male mice and not like that of normal female mice. Furthermore, the initial stimulating effect of testosterone on the respiration of the liver slices and diaphragm of young male mice required the presence of glucose(4), and was much greater than that observed in the tumor bearing female mice.

The decrease in oxygen consumption of the tissue slices does not occur until the concentration of testosterone greatly exceeds physiological levels as calculated from renotrophic and androgenic effects in the intact mouse(5). A relatively high concentration, 0.28 micromolar, of testosterone, however, did not affect the oxygen consumption of primary Brown-Pearce epithelioma (unpublished).

Testosterone has been reported to decrease the respiration of rat brain(6-8), striated

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[*] Present address: Oklahoma Medical Research Institute, Oklahoma City, Okla.

[†] The studies were carried out in 1939-1940 before the Department of Vital Economics was merged with the Department of Physiology.

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[‡] Testosterone was provided by Ciba Pharmaceutical Products, Inc.

[¶] Mice were generously provided by Dr. Earle B. Mahoney, Department of Surgery.

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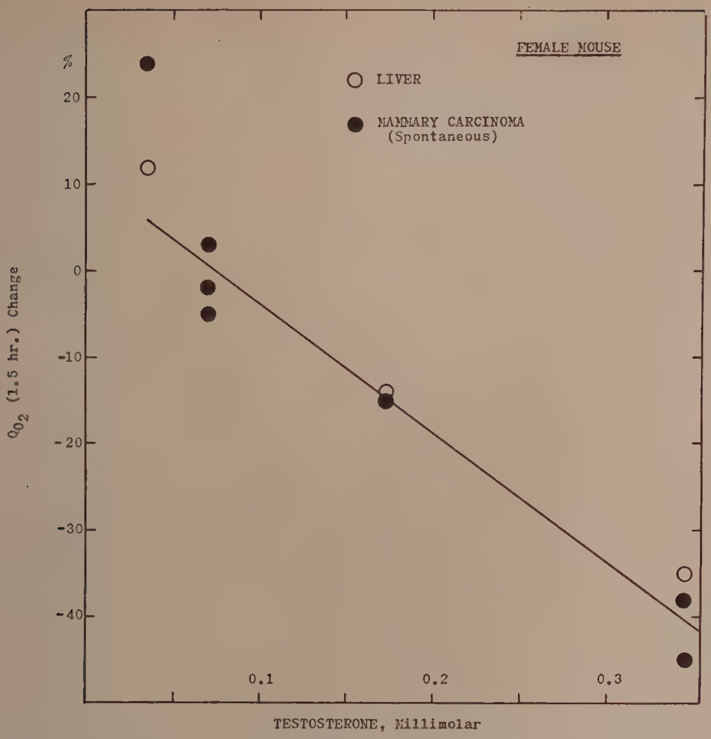

FIG. 1. Effect of testosterone on respiration of liver and spontaneous mammary carcinoma slices from female mice.

muscle(6,7), liver(8,9), and kidney(8,9) preparations. Moreover, the degree of inhibition has been correlated(9) with the quantity of testosterone added.

Summary. The oxygen consumption of liver and spontaneous mammary tumor slices from female mice was initially slightly increased and then progressively decreased by increasing concentrations of added testosterone.

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9. Fondal, E., and Kochakian, C. D., unpublished.

Received July 3, 1951. P.S.E.B.M., 1951, v77.

Distribution of Radioactive Colchicine in Some Organs of Normal and Tumor-bearing Mice.* (18886)

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A small quantity of radioactive colchicine labelled with carbon-14 was first prepared by the use of biosynthesis technics in this laboratory during the past year. The limited quantities of radioactive colchicine obtained thus far by this method have been employed in experiments on the distribution of this drug in the tissues of normal and tumor-bearing mice. Radioactive colchicine makes it possible to determine with greater precision the presence of a small amount of colchicine in tissues, or in a conglomerate of cells. The results presented herein indicate that the distribution of colchicine in some tissues of tumor-bearing animals is markedly altered from the normal pattern.

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* This work was done under a contract between the AEC and the University of Chicago. It was aided in part by a grant from the Stewart Memorial Cancer Fund.

† On leave-of-absence from the Cancer Laboratories of the Hebrew University, Jerusalem.

Methods. Radioactive colchicine randomly labelled with carbon-14 and containing a specific activity of 40,000 counts/min/mg or 27 microcuries/g was obtained from *Colchicum autumnale* corms which were made radioactive by the biosynthetic procedures developed in this laboratory(1). The alkaloid was extracted and purified by modifications of the available methods devised by one of us (W) to make possible the handling of minute quantities of starting material and of purified material(2). For administration to mice the colchicine was dissolved in distilled water (1 mg/ml) and injected subcutaneously at a dose of 1 ml per mouse. This dose produced diarrhea after 2 to 3 hours and death ensued after 8 to 12 hours. Throughout these experiments C-57 black mice secured from the Bar Harbor Laboratory were used. The animals weighed 20-25 g. No distinction was made as to sex but no pregnant animals were used.

The tumor employed was sarcoma 180, and was implanted in the scapular region. In our laboratory the implant was observable after 5-7 days, and reached its development without necrosis after 10-15 days; at which time the animals were sacrificed. All of the animals used for distribution studies were sacrificed 4 hours after injection. The organs were removed, washed in saline to remove blood, dried between filter paper, weighed and homogenized in a Potter and Elvehjem homogenizer. The homogenates (10%) were then extracted with 70% ethanol and filtered. The filtrate was evaporated to a small volume, diluted with water and extracted with petroleum ether. The aqueous fraction was then extracted with chloroform and this extract was then evaporated to dryness and taken up in chloroform C.P. (0.7% ethyl alcohol). This extract was then allowed to pass through an alumina column. The resulting eluate contained colchicine while other substances were adsorbed on the column. The identification of the colchicine was accomplished by the use of a polarographic method(2). Equal parts of each sample were analyzed using an internal Geiger counter of special construction(3).

The data so obtained are expressed as counts/min per gram of tissue and as percentage of total counts found in the animals.

Results and discussions. Our reasons for performing this study were to (a) determine whether tumor tissue has a tendency to accumulate colchicine and (b) to ascertain whether the presence of tumors in mice brings about changes in the distribution pattern of colchicine.

It was found that labelled colchicine was not present in blood, brain, muscle and heart 4 hours after injection into normal mice. Table I shows the distribution of colchicine in some organs of normal mice C-57. Colchicine was found in the liver in very small quantities. The intestine, kidney and spleen accumulated the drug in considerable amounts. It is known from the papers of Jacobj(4) and more recently Brues(5) that most of the colchicine is excreted in the feces and urine during the first hours after injection. The variability of the percentage of colchicine in the kidney and intestine might be explainable on the basis of differences in excretion.

In the tumor-bearing mice it was also found that colchicine is not present in blood, brain, muscle and heart.

In Table II the uptake of colchicine in some organs of tumor-bearing mice is shown. In the intestine and kidney the uptake of colchicine was no smaller than was observed in

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1. Geiling, E. M. K., Kelsey, F. E., McIntosh, B. J., and Ganz, A., *Science*, 1948, v108, 558.
2. Walaszek, E., in press.
3. Kelsey, F. E., *Science*, 1949, v109, 2840, 566.
4. Jacobj, C., *Arch. Exp. Path. Pharmakol.*, 1890, v27, 119.
5. Brues, A. M., *J. Clin. Invest.*, 1950, v21, 646.

TABLE I. Distribution of Colchicine in Normal Mice. One column represents the number of counts over background per g of tissues. The other column represents the same values expressed as % of the total counts taken in a mouse.

	Intestine		Kidney		Liver		Spleen	
	cpm/g	%	cpm/g	%	cpm/g	%	cpm/g	%
1.	50	2.22	54.1	30	18.5	10.2	67.2	37
2.	21.1	11.7	92.9	50.6	6.9	3.8	64.2	33.9
3.	64.8	26	78.5	31.4	3.3	1.3	104.4	41.3
4.	74.5	26.6	82.1	29.3	21.1	7.5	103	36.6
5.	67	26.4	53.6	21.4	29.1	10.9	104.2	41.8
6.	23.2	12.8	33.5	18.6	49.4	27.4	74	41.2

TABLE II. Distribution of Colchicine in Tumor Bearing Mice. One column represents the number of counts over background per g of tissue. The other column represents the same values expressed as % of the total counts taken in a mouse.

	Intestine		Kidney		Liver		Tumor		Spleen	
	cpm/mg	%	cpm/mg	%	cpm/mg	%	cpm/mg	%	cpm/mg	%
1.	174	40.4	116	27	76.8	17.9	71.1	17.7	0	
2.	72	55.4	35.6	27.4	13.5	10.3	12.1	6.9	0	
3.	118.2	48.2	74.9	31.2	29.2	12.1	26.2	17.5	0	
4.	79.3	33	58.6	24.4	51.6	21.5	50.1	21.1	0	
5.	57.4	27.7	43.2	21.4	48.9	24.4	53.7	26.5	0	
6.	104.2	40	97.3	37	29.1	11	31.7	12	0	
7.	74.6	31	36.1	18.9	90.4	33.5	40	16.6	0	
8.	98	49	36.1	23.7	20	10	29.7	18.3	0	

normal mice. Liver and tumor contained approximately the same amount of colchicine, which amounted to 10 to 26 percent of the total counts/min/g found in the mice. The colchicine is taken up by sarcoma in an amount sufficient to produce its specific action on that tissue.

It was found that in the spleen of normal animals the accumulation of colchicine was from 33 to 42% of the injected dose. However, in the spleen of tumor-bearing animals no colchicine could be detected. Concentration of colchicine in the spleen of normal mice presupposes intervention of the reticuloendothelial system. To test this hypothesis the reticuloendothelial system in normal mice was blocked by an injection of India ink. The radioactive colchicine was injected 2 or 3 or 4 hours later, and the spleen was removed 4 hours after and analyzed for colchicine. The amount of colchicine found was approximately the same as found in a mouse not injected with India ink.

Summary. 1. Labelled colchicine was detected only in some organs of normal and tumor-bearing mice. 2. The uptake of colchicine in sarcoma 180 is as high as the uptake of liver. 3. Spleen is one of the organs having the highest concentration of colchicine in normal mice. 4. It was found that there was no colchicine in the spleen of tumor-bearing mice.

Received July 6, 1951. P.S.E.B.M., 1951, v77.

Effect of Pentobarbital on the Toxicity of Several Aliphatic and Heterocyclic Quaternary Ammonium Compounds.* (18887)

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In the course of an investigation on the acute toxicity of an homologous series of aliphatic and heterocyclic quaternary ammonium derivatives in mice, tremors and generalized convulsions characteristic of central stimulation were observed. Pentobarbital (Nembutal) anesthesia was found to protect animals against the toxicity of the majority of these compounds. Certain compounds containing specific groups in the molecule were not affected by the Nembutal anesthesia. This report is concerned with (a) the influence of structure on the toxicity of several aliphatic and heterocyclic quaternary ammonium derivatives, and (b) the influence of Nembutal on the toxicity of these compounds.

Methods. Male mice (Maple Grove Rabbitry, Springfield, Mo.) weighing 18 to 23 g were used for these studies. All injections were given by the intraperitoneal route. Nembutal, 33 or 66 mg/kg, was administered 15 minutes prior to the challenge with the quaternary ammonium compound. The high dose of Nembutal invariably resulted in deep narcosis; at the lower dose the anesthesia was light.

Three or more dose levels of each quaternary ammonium derivative were tested in both the control and the Nembutal pretreated groups. Although the majority of animals died within one hour, observations for mortality were made for 24 hours. Two experiments were usually run on each compound, and the data were combined for statistical analysis. The method of Litchfield and Wilcoxon(2) was used for estimating the LD_{50} values and factors (f) for fiducial limits. Protection was considered to have occurred when the LD_{50} of the pretreated group was increased significantly ($P = 0.05$) above that of the control group.

Results and discussion. Among the aliphatic compounds there is a progressive increase in toxicity with an increase in the chain length from 2 to 4 carbons (Table I). This is illustrated by the straight-chain compounds, No. 1, 2, 3, and 4, and also by the branched-chain pair, No. 6 and 7. Triethylbutylammonium (No. 3) is significantly more toxic than its isomer, diethyldipropylammonium (No. 2), which gives further indication that the length of the alkyl chain rather than the size of the molecule is important in altering the toxicity. In general, compounds with branched alkyl chains (No. 5, 6, 7, and 8) are relatively less toxic than their straight-chain homologues. The cyclic, piperidinium, derivatives have about the same order of toxicity as their aliphatic analogues. The dose-response curves for all compounds are essentially parallel, indicating a similar mechanism of action.

Pretreatment with 66 mg/kg of Nembutal resulted in a significant reduction in toxicity for the majority of compounds (Table I) but did not alter the slopes of the dose-response curves. However, 4 compounds were not antagonized by Nembutal anesthesia (No. 6, 7, 10 and 11). These have the following structural features in common: (a) 2 of the alkyl groups or the piperidinium ring have methyl substitutions on the carbons alpha to the nitrogen, and (b) at least one of the 2 additional substituents on the nitrogen is an ethyl group. Pretreatment with 33 mg/kg of Nembutal failed to reduce the toxicity of any of the compounds in Table I.

Summary. In a study of an homologous series of aliphatic quaternary ammonium and piperidinium compounds in mice, it was found that an increase in the chain length of 2 alkyl groups from 2 to 4 carbons results in an increase in the acute toxicity. Compounds with

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* These compounds are members of a series synthesized in the laboratories of G. D. Searle & Co. by Richard A. Robinson(1).

1. Robinson, Richard A., to be published.

2. Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharm. and Exp. Therap.*, 1949, v95, 99.

branched chains are relatively less toxic than compounds with straight chains. Piperidinium derivatives have the same range of toxicity as their aliphatic analogues. Administration of an anesthetic dose of Nembutal (66 mg/kg), 15 minutes prior to an injection of the

TABLE I. Reduction of Toxicity of Quaternary Ammonium Derivatives by Nembutal Anesthesia in mice.

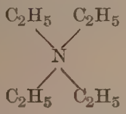
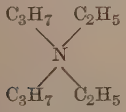
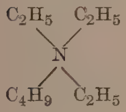
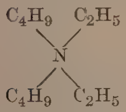
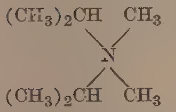
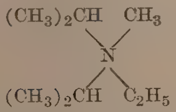
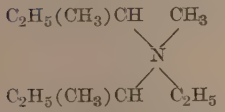
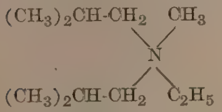
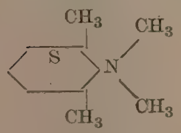
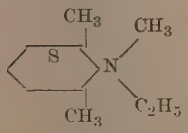
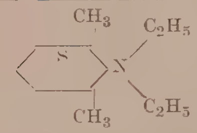
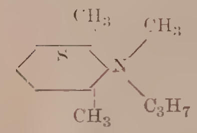
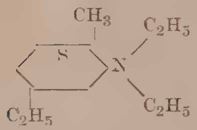
	Formula			Control (C)			Nembutal (N), 66 mg/kg			
No.	Cation	Anion	Mol wt of base	LD_{50}, mM/kg	f*	No. of animals	LD_{50}, mM/kg	f*	No. of animals	Protection ratio, N/C
1.		Br	130.3	.300	1.18	60	.666	1.23	75	2.22†
2.		Br	158.3	.221	1.23	42	.351	1.28	53	1.59†
3.		Br	158.3	.148	1.26	48	.237	1.28	48	1.60†
4.		Br	186.3	.107	1.24	72	.219	1.27	78	2.05†
5.		Br	130.3	.352	1.19	48	.590	1.27	48	1.68†
6.		Br	144.3	.480	1.38	18	.325	1.89	24	.68
7.		Br	172.3	.215	1.21	42	.221	1.20	48	1.03
8.		I	172.3	.318	1.22	42	.493	1.20	48	1.55†
9. ‡		Cl	142.2	.314	1.25	48	.574	1.37	48	1.83†
10.		Br	156.3	.254	1.18	24	.254	1.42	24	1.00

TABLE I (Continued)

No.	Formula: Cation	Formula: Anion	Mol wt of base	Control (C): LD_{50}, mM/kg	Control (C): f*	Control (C): No. of animals	Nembutal (N), 66 mg/kg: LD_{50}, mM/kg	Nembutal (N), 66 mg/kg: f*	Nembutal (N), 66 mg/kg: No. of animals	Protection ratio, N/C
11.		Br	170.3	.100	1.27	48	.114	1.24	48	1.14
12.		Br	170.3	.220	1.22	48	.304	1.28	48	1.38†
13.		Br	184.3	.104	1.32	42	.189	1.33	48	1.82†

* The "f" of a term is the factor by which the term is multiplied and divided to determine its confidence limits at P = 0.05.

† Significant increase in LD_{50}.

‡ The S in the ring indicates complete saturation (piperidinium ring).

quaternary ammonium derivative, reduces the toxicity of all but 4 compounds. These 4 compounds have methyl branches on 2 of the alpha carbons and at least one of the other substituents on the nitrogen is an ethyl group.

Received July 10, 1951. P.S.E.B.M., 1951, v77.

Inhibition of Hemagglutination of Columbia-SK Virus by Human Polio-convalescent Sera.* (18888)

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Discovery of the fact that Columbia-SK virus causes hemagglutination of sheep red cells(1) permits use of the phenomenon to determine the presence of hemagglutination-inhibitory antibodies in human sera against this virus and related strains (MM, EMC, Mengo, F). A standard method was recently developed by Gard and Heller(2) for the assay of such antibodies against MM virus in the sera of patients suffering from various neurotropic infections. When applied to a series of 569 human sera this test yielded 12.5% strongly positive reactions in a group of 384 sera collected from patients with a diagnosis of paralytic or non-paralytic poliomyelitis, aseptic meningitis or encephalitis. By contrast, only 0.7% similarly positive reactions were encountered in a control group of 185 sera, including 39 samples from patients with various diseases showing no involvement of the central nervous system and 146 samples from healthy individuals. None of the positive human sera were capable of neutralizing the virus in mice. We have undertaken to repeat these experiments with a small number of convalescent sera from cases of poliomyelitis or of encephalitis and with "normal" human sera, using Col-SK virus as the test agent and employing a somewhat different but comparable technic(3). The results of this investigation are reported herewith.

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* Aided by a grant from the Sister Elizabeth Kenny Foundation.

1. Hallauer, C., *Proc. IVth Internat. Congr. Microbiol.* (July 1947), Copenhagen, 1949, p257; Verlinde, J. D., and de Baan, P., *Ann. Inst. Pasteur,* 1949, v77, 632; Olitsky, P. K, and Yager, R. H., Proc. Soc. Exp. Biol. and Med., 1949, v71, 719.

2. Gard, S., and Heller, L., Proc. Soc. Exp. Biol. and Med., 1951, v76, 68.

Materials and methods. The poliomyelitis sera were obtained in the Fall of 1950 from paralytic and non-paralytic cases during the acute or subacute stage of their illness; the encephalitis sera were from patients convalescent from an attack of Jap B encephalitis in 1950.† The "normal" sera were collected from healthy donors at the Blood Bank of the Presbyterian Hospital, New York City. Prior to their use in the hemagglutination-inhibition test all sera had been absorbed with packed sheep red cells (1 hour at 37°C and overnight in the icebox) in order to remove any heterophile hemagglutinins. This procedure was adopted routinely because a majority of the polio-convalescent sera contained abnormally high levels of cold agglutinins against sheep erythrocytes(4). The virus was obtained from the brains of paralyzed passage mice. Several freshly harvested viral brains were pooled and ground with veronal-buffer(5) so as to make a 10% suspension. This suspension was lightly centrifuged and the supernatant fluid further diluted serially with the same diluent. Concurrent with the inhibition tests the hemagglutinating activity of each virus pool was titrated by combining 0.2 cc of the various virus dilutions with 0.2 cc of a 0.25% suspension of washed sheep red cells in veronal-buffer and adding 0.2 cc of veronal-buffer to each tube. The sheep cells had previously been collected in Alsever's solution and were stored in the icebox for a period not exceeding 2 weeks. The hemagglutinating system was placed in the icebox and the reactions were read the following morning. With the use of veronal-buffer and the choice of suitable sheep erythrocytes viral hemagglutination resulted not in pattern, but in compact aggregation of cells which could be broken up only after shaking the tubes. No such reactions were obtained with normal mouse brain. The smallest amount of virus which produced clumping, clearly visible by macroscopic observation, was designated as one hemagglutinating unit. Under the conditions of our tests the hemagglutinating activity of the several virus preparations was remarkably constant, one unit usually being equivalent to a 1:640 to 1:1280 brain dilution. For the hemagglutination-inhibition test the absorbed sera were serially diluted (1:5-1:1280) with veronal-buffer and 0.2 cc of the various serum dilutions were mixed with a fixed dose of virus, *i.e.* 0.2 cc of a 1:160 viral brain dilution calculated to equal 4 or 8 hemagglutinating units. The serum-virus mixtures were held for 1 hour at room temperature and 0.2 cc of a 0.25% sheep red cell suspension was added to each tube. The reactions were read after overnight refrigeration together with those of the virus titration. Controls with normal and immune monkey sera, as well as cells alone in veronal-buffer, were included in each test. Whereas normal monkey sera never inhibited viral hemagglutination, many of the human sera gave partial hemagglutination inhibition. This necessitated a somewhat arbitrary evaluation of the results in order to separate definitely positive reactions from reactions of doubtful significance. A standard code was finally adopted under which absence of hemagglutination was considered "negative" when it occurred only in serum dilutions below 1:10, "questionable" when it occurred only in serum dilutions below 1:20,

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3. Horvath, B., and Jungeblut, C. W., *Fed. Proc.*, 1951, v10, 359.

† Our thanks are due to Drs. M. A. Stevens of the Jersey City Medical Center and E. J. Huenekens of the Kenny Institute, Minneapolis, Minn., for providing the poliomyelitis sera. We are also indebted to Dr. Masami Kitaoka of the National Institute of Health of Japan, Tokyo, Japan, for kindly sending us the encephalitis sera.

4. Jungeblut, C. W., Unpublished data.

5. Mayer, M. M., Croft, C. C., and Gray, M. M., *J. Exp. Med.*, 1948, v88, 427.

TABLE I. Code for the Interpretation of Hemagglutination-Inhibition of Col-SK Virus (4-8 H.U.) by Human Sera.

Sera	Serum dilutions							Result
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	
Human serum 1	*1*	4	4	4	4	4	4	Negative
,, ,, 2	0	*1*	4	4	4	4	4	,,
,, ,, 3	0	0	*1*	4	4	4	4	Questionable
,, ,, 4	0	0	0	*1*	4	4	4	Positive
,, ,, 5	0	0	0	0	0	*1*	4	,,
Normal cynomolgus serum	4	4	4	4	4	4	4	Negative
Immune Col. SK cynomolgus serum	0	0	0	0	0	0	1	Positive

0 = no hemagglutination, 1 (2-3) = partial hemagglutination, 4 = complete hemagglutination.

TABLE II. Hemagglutination-Inhibition of Col-SK Virus by Sera from Normal Persons and by Sera from Acute Cases of Poliomyelitis or Japanese B Encephalitis.

Sera	No.	Hemagglutination-Inhibition		
		Negative	Questionable	Positive
		%	%	%
Normal sera (New York City)	80	74 (92.5)	4 (5)	2 (2.5)
Poliomyelitis sera (Jersey City)	40	39 (80)	4 (8)	6 (12)
Poliomyelitis sera (Minneapolis)	8	4 (50)	1 (13)	3 (37)
Total poliomyelitis sera	57	43 (75)	5 (9)	9 (16)
Jap B encephalitis sera (Tokyo)	12	12	0	0

and "positive" when it occurred in serum dilutions of 1:20 or above. An illustration of the basic principle is given in Table I.

Results. The results of the inhibition tests (Table II) show that a small percentage (16%) of the polio-convalescent sera gave strongly positive reactions, whereas the Jap B encephalitis sera were uniformly negative. The control series of "normal" sera showed an insignificant incidence (2.5%) of strongly positive reactions. Of the 14 positive or questionable poliomyelitis sera 5 were positive (Index 100-1000) and 9 were negative when tested for neutralization of Col-SK virus in mice. None of the Jap B encephalitis sera had neutralizing antibodies for Col-SK virus but all, except 2, neutralized Y-SK virus.

Discussion. Our observations are in essential agreement with those reported by Gard and Heller(2) from Sweden and with the experience of Keller(6) in Germany. They indicate that a small percentage of sera from poliomyelitis patients, collected during the early stage of their illness, are capable of bringing about definite inhibition of hemagglutination of sheep red cells by Col-SK virus. This property was not destroyed by heating the sera for ½ hour at 56°C. Similarly positive reactions were absent in a small group of sera from patients convalescing from another neurotropic virus infection (Jap B encephalitis) and were only found sporadically among sera from healthy individuals. Yet, the data do not permit the assertion that this reaction reflects a specific response to infection with this virus because serological reactivity was not followed through the course of the disease. Moreover, hemagglutination-inhibition and neutralization reactions, though occurring together in some sera, showed no constant relationship. It is impossible, at present, to say whether hemagglutination-inhibition and neutralization measure different antibodies in the human sera or whether the discrepancy between the *in vitro* and *in vivo* methods is due to quantitative differences in the operation of the same antigen-antibody reaction. Nevertheless, the data are in general agreement with our earlier observations, according to which significant neutralizing

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6. Keller, W., Personal communication.

antibody levels against Col-SK virus could be demonstrated in 28 (21%) of 132 sera from patients, residing in Greater New York, who had been diagnosed as paralytic or non-paralytic poliomyelitis(7). Recent studies of poliomyelitis occurring in certain areas of Mexico revealed an even higher frequency of neutralizing and hemagglutination-inhibitory sera among cases and contacts(8). Barring any other plausible explanation for such differences, the possibility should be considered that geographic variation in the distribution of the virus may be a factor concerned with the selectivity of the serological results.

Summary and conclusions. In a series of 57 sera collected from poliomyelitis patients during the acute or subacute stage of their illness, 9 (16%) gave strongly positive reactions, 5 (9%) gave questionable reactions and 43 (75%) gave negative reactions when tested for the presence of hemagglutination-inhibitory substances against Col-SK virus. All of 12 sera from patients convalescing from Jap B encephalitis gave negative reactions. In a control group of 80 sera from healthy individuals 2 (2.5%) gave positive reactions, 4 (5%) gave questionable reactions, and 74 (92.5%) gave negative reactions. Of 14 positive or questionable poliomyelitis sera, 5 contained significant neutralizing antibody levels against Col-SK virus as determined by mouse test. The significance of these findings is discussed.

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7. Jungeblut, C. W., *Arch. Ped.,* 1950, v67, 519.
8. Jungeblut, C. W., and Bautista, G., In preparation.

Received June 27, 1951. P.S.E.B.M., 1951, v77.

Isolation of an Active Polysaccharide Fraction from Plague Organisms.* (18889)

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An attempt to isolate an active polysaccharide from the supernatant of the Haffkine plague vaccine was made by Shrivastava(1). The substance reacted with antiplague horse and rabbit sera but the yield from a large quantity of supernatant (200 litres) was extremely low. The protein-free casein hydrolysate medium of Mueller and Johnson(2) as modified by Seal and Mookerji(3) offered an opportunity to reopen the investigation. The results of the trials to isolate the polysaccharide fractions from 3 sources, namely, (a) supernatant of the Haffkine plague vaccine prepared in the casein hydrolysate broth, (b) bacterial debris of the same vaccine, and (c) specific soluble protein of plague bacilli (Seal) (4) are reported below.

(A) *Isolation from the supernatant of the Haffkine plague vaccine. Strains used:* 1) Virulent plague strains 337/L and 139/L obtained from human plague cases; 2) Avirulent protective plague strain 53H/av originally obtained from a human plague case and made avirulent in the laboratory; 3) Avirulent non-protective plague strain TRU isolated by Schutze(5) from Java human strain Tjiwidej R(4) *Past-pseudotuberculosis* strains, PR/I and PR/IV, obtained from the National Type Cultures Laboratory, London.

Methods. Seven liters of bacteria-free filtrate of 3 weeks' growth, at 28°C, of the virulent plague strain 337/L in casein hydrolysate broth at pH adjusted to 7.2-7.3, was reduced to 1/7 of its volume *in vacuo* over a water bath maintained at 45°-47°C. The material was clarified by centrifuging and the Molisch positive washings of the residue and water were added to bring the volume to 1500 ml, followed by 40 g of sodium acetate, 9 ml of glacial acetic acid and 3 volumes of alcohol and the mixture was left overnight at room temperature. The precipitate thus formed was recovered by centrifuging, taken up in 200 ml water and mixed with 4 ml of glacial acetic acid and 10 ml of saturated solution of sodium acetate. The clarified filtrate of this material, which was strongly Molisch and faintly biuret positive, was precipitated with one half volume of absolute alcohol at room temperature and its supernatant, left after removal of the precipitate, was also similarly treated. The precipitates thus obtained were pulled together and purified by repeated treatment with glacial acetic acid, sodium acetate solution and one half volume of absolute alcohol and finally dried in a desiccator over P_2O_5. The total yield was 23.4 mg.

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* This work was originally carried out in 1942-43 at the Haffkine Institute, Bombay.

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Serological behavior. A solution of the substance diluted to contain 1 mg/ml was put up for ring-precipitation test with 12 antisera produced in rabbits against various plague and pseudotuberculosis antigens given I.V. The results are given in Table I.

The results given in Table I show that the polysaccharide fraction isolated from the virulent plague bacillus reacted with antisera raised against both virulent and avirulent protective plague strains *viz.*, 337/L and 53/Hav respectively. This activity was retained by these sera even after absorption with the non-protective plague strain TRU or the pseudotuberculosis strain PR/I, but the substance gave completely negative reaction with antisera produced against 337/L boiled suspension, TRU, PR/I and the protein fraction P(½-⅓). Thus the substance was related to the specific soluble protein or Antigen I of plague bacillus (corresponding to the envelope antigen of Schutze)(5), and not to its somatic part or Antigen II of plague bacillus which corresponds with the antigens used for producing the antisera giving negative reactions (Seal)(4). Consequently attempts to isolate this substance from the non-protective plague and pseudotuberculosis strains which are related antigenically to the somatic part of plague bacillus, failed.

TABLE I. Results of Ring-Precipitation Test of Plague Polysaccharide (1 mg/ml) Against Various Absorbed and Unabsorbed Antiplague and Anti-pseudotuberculosis Rabbit Sera.

Antisera	Result
1. 337/L (live)*	2+
2. ” abs with TRU	1+
3. ” ” ” PR/I	1+
4. ” (boiled ½ hr)	0
5. TRU (live)	0
6. PR/I (live)	0
7. 53/Hav (live)	2+
8. ” abs with TRU	1+
9. ” ” ” PR/I	1+
10. Lauto P⅓ (plague protein)	2+
11. L_5 P⅓ (” ”)	1+
12. L_5 P½-⅓ (” ”)	0

* Notes in parentheses are description of antigens used for the production of antisera, No. 10-12 being the specific soluble protein fractions of plague bacillus (Seal)(4).

(B) *Isolation from the bacterial debris of the Haffkine plague vaccine.* Fifty ml of bacterial debris, obtained by centrifuging 3½ weeks' growth, at 28°C, of virulent plague strain 337/L in casein hydrolysate broth, was hydrolysed with 6% H_2SO_4 over water bath under reflux for 5 hours. The solid part of the hydrolysate was removed in the centrifuge and the liquid portion neutralized to Congo-red paper with sodium acetate, boiled with animal charcoal and filtered. Osazone crystals were prepared from this filtrate and purified according to the method used by Linton *et al.*(6). The crystals, as seen under the microscope, resembled *Arabinose* osazone (Fig. 1) and gave a melting point of 166°-168°C, the same that of known *Arabinose* osazone. Similar osazone crystals were also prepared from the debris of another virulent plague strain 139/L and avirulent protective plague strain 53/Hav, while TRU strain showed only a few stray crystals but none could be isolated from the debris of pseudotuberculosis, PR/I and PR/IV.

(C) *Isolation from the specific soluble pro-*

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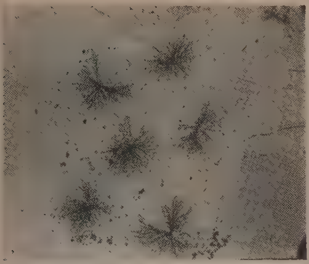

FIG. 1. Microphotograph of osazone crystals obtained from the virulent plague strain, 337/L.

tein of plague bacilli. The same method as used in the case of bacterial debris was employed for preparing osazone crystals from the specific plague proteins, L auto P1/3 and $L_5P1/3$. The same type of crystals in a purer form was obtained in both of them, the melting point being 166°-168°C.

Comments. In the attempt to isolate polysaccharide from the supernatant of the Haffkine plague vaccine prepared in the enriched casein hydrolysate broth a very small quantity (23.5 mg) could be isolated from 7 liters of the vaccine. From the nature of its chemical and serological reactions it seems that the substance is intimately associated with the specific soluble proteins for the protective plague strains. This has been confirmed by isolating an arabinose-like osazone (melting point 166°-168°C) from the plague proteins, $L_5P1/3$ and Lauto P1/3. The same osazone crystals were found in the protected plague strains 139/L and 53/Hav but not in the avirulent non-protective plague strains TRU or in the pseudotuberculosis strains, PR/I and PR/IV. These findings lend support to the view that the specific protective substance of plague bacilli is a polysaccharide-protein complex, probably a nucleoprotein.

Summary. A polysaccharide yielding osazone resembling that of *Arabinose* with a melting point of 166°-168°C has been isolated from the supernatant of the Haffkine plague vaccine as well as from the specific soluble protein and, to a less extent, from the bacterial debris of both virulent and avirulent protective plague strains. It is absent in non-protective avirulent plague and pseudotuberculosis organisms. The protective substance of the plague bacillus is probably a polysaccharide-protein complex.

This work was carried out under the active support of Major-General Sir Sahib Singh Sokhey, the late Director, Haffkine Institute, Bombay.

Received May 11, 1951. P.S.E.B.M., 1951, v77.

Serum Iron and Hemoglobin Values of 275 Healthy Women.* (18890)

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(Introduced by C. E. Georgi.)

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The importance of serum iron in the intermediary metabolism of iron is recognized. Many investigators have reported serum iron values for women and these values range from a low of 30 μg % as reported by Eckerström (1) to a high of 210 μg % as reported by Vahlquist(2). There is some variation in the range of values which is considered by different investigators to be normal for women. Dahl(3), who has studied the greatest number of subjects recently, suggests 70 μg to 140 μg % as the normal range of serum iron values for women; Moore *et al.*(4) suggest the wider range of 50 μg to 180 μg %; and, although Vahlquist(2) considers 50 μg % to be the lower limit of normality, he states that the upper limit must be placed considerably above 200 μg %. Some investigators have found that menstruation affects the serum iron level. Dahl(3) states that serum iron values were lower for women before the menopause than after. Within a single menstrual cycle he found the highest values shortly before menstruation and the lowest during and immediately after. Powell(5) confirmed this trend but Cartwright *et al.*(6) and Brøchner-Mortensen and Olsen(7) found no relationship between serum iron values and different times during a menstrual cycle. Age is another factor which has been found to affect serum iron levels in some cases. Eckerström (1) compared mean serum iron values of 100 men and women, 70 to 94 years old, with values of adults studied by Vahlquist(2) and found the elderly subjects had lower serum iron values. Brøchner-Mortensen and Olsen (7), however, reported no dependency of serum iron levels upon age. Cartwright *et al.* (6) agreed with this finding although only one-third of their 43 subjects were older than 30 years.

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* Contribution No. 3, Subproject—"The Nutritional Status and Dietary Needs of Older People" of the North Central Cooperative Project NC-5 "Nutritional Status and Dietary Needs of Population Groups." In cooperation with the Bureau of Human Nutrition and Home Economics, U. S. Department of Agriculture. Published with the approval of the Director as paper no. 537 Journal Series, Nebraska Agric. Exp. Station.

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In a study of the nutritional status of healthy women in this laboratory it seemed pertinent to include an evaluation of serum iron levels.

Procedure. Serum iron and hemoglobin values were determined for 275 healthy women who were between the ages of 17 and 86 years and were eating self-chosen diets. The 17- to 25-year-old women were college students, the 30- to 86-year-old women were homemakers and business and professional women. About half of the subjects had children, the number ranging from one to 7. With few exceptions all subjects were above average in socio-economic status. Fasting blood samples, obtained in the early morning by venipuncture, were used for the determination of serum iron and hemoglobin. Samples were not taken during the first 3 days of menstruation. A protein-free extract was prepared by precipitation and heating of the serum as described by Kitzes *et al.*(8). Iron was determined in this extract by the method of Pohle *et al.*(9). The optical density of the colored complex formed by 1, 10-phenanthroline and the reduced iron was measured in a Beckman spectrophotometer for which the extinction coefficient of iron had been determined. Precautions were taken to avoid hemolysis of the whole blood. Glass-distilled water and iron-free reagents were used. Hemoglobin was converted to oxyhemoglobin according to the method of Todd and Sanford (10) using 0.1% Na_2CO_3 and the depth of color determined in a Sheard-Sanford photelometer which had been calibrated with blood of a known oxygen capacity.

Results. Serum iron and hemoglobin values were sorted into groups according to the ages of the women. The mean values and standard deviations for serum iron and hemoglobin are given in Table I for each of the 7 age groups and for the combined groups.

Serum iron values ranged from 33 μg to 221 μg % and the mean value for all subjects was 116 ± 33 μg %. The youngest group, 17 to 25 years, had a mean serum iron value of 125 μg %. With the exception of 122 μg % for the 50 to 59 year group the mean values for each successive age group declined to 95 μg % for the 70 to 79 year group, then rose slightly to 101 μg % for the oldest group, 80 to 86 years. Analysis of variance indicated that a significant difference in serum iron values existed among the different age

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TABLE I. Serum Iron and Hemoglobin Values of Women in This Study.

Age groups	No. of subjects	Mean age, yr	Serum iron		Hemoglobin	
			Mean value	Std dev	Mean value	Std dev
			µg %		g %	
17-25	52	20	125	31	13.2	.8
30-39	35	34	119	40	13.2	.9
40-49	63	44	113	35	13	.9
50-59	64	55	122	30	13	.8
60-69	32	63	110	34	13.4	.9
70-79	22	74	95	21	13.4	.8
80-86	7	83	101	17	13.5	1.2
All	275		116	33	13.2	.9
Range		17-86	33-221		10.4-16.5	

TABLE II. Serum Iron and Hemoglobin Values Related to Menopause.

Stage	No. of subjects	Serum iron		Hemoglobin	
		Mean value	Std dev	Mean value	Std dev
		µg %		g %	
Pre-menopause	30	114	37	13	.8
During "	23	105	36	13.2	.9
Post "	18	115	22	12.9	.7

groups ($F = 3.19$; $F_{.01} = 2.90$). The regression coefficient of serum iron on age was —.343. The t test showed this coefficient to be significant at the 1% level of probability ($t = 3.014$; $t_{.01} = 2.592$).

The mean hemoglobin value for all subjects was 13.2 ± 0.9 g % with a range of 10.4 g to 16.5 g %. No significant difference among the mean hemoglobin values existed for the different age groups nor was there any correlation between the hemoglobin values and the serum iron. Nineteen of the women had hemoglobin values below 12.0 g %, or from 10.4 to 11.9 g %. These low values indicated anemia, although medical records did not show any other symptoms of abnormality. The mean serum iron and hemoglobin values for the 19 women were 107 µg % and 11.6 g %, respectively. When the serum iron values of these women were omitted from the entire group, the mean value of the remaining 256 women was 117 ± 33 µg %, as compared with 116 ± 33 µg % for the 275 women. The range of serum iron values and of ages remained unchanged. Omission of the hemoglobin values of these 19 women from the group raised the mean hemoglobin value from 13.2 ± 0.9 g % to 13.3 ± 0.8 g %.

The relationship of serum iron values to menopause was investigated. The women who were going through the menopause ranged in age from 40 to 52 years. Therefore, the serum iron and hemoglobin values for all women in this age range were grouped on the basis of their relation to the menopause; that is, pre-menopause, during menopause, or post-menopause, and the results are presented in Table II. The mean serum iron values were 114 ± 37, 105 ± 36, and 115 ± 22 µg %, respectively. An analysis of variance indicated that no significant difference existed among these values.

Discussion. Serum iron values of 33 µg to 221 µg % found in this study constitute a wider range than has been considered normal by many investigators. Five per cent of the women had values below 68 µg % and 5% had values above 180 µg %. Careful scrutiny was given to the records of the thorough medical and physical examinations of these women and no abnormalities were observed which could account for either the low or the high values for serum iron. In only one case was a low serum iron value, 58 µg %, accompanied by a low hemoglobin value, 10.7 g %.

In view of the many physiological factors which normally influence the level of serum iron in the blood a wide range of normal values is not surprising. These factors include the rate of iron absorption, the balance between iron entering and leaving the tissues, the size of storage depots, and the rate of the production and breakdown of hemoglobin. Abnormal serum iron values may be caused by severe blood losses and certain pathological conditions.

No explanation is apparent for the increased mean serum iron value of 122 μg % for the 50 to 59 year group as compared with 113 μg % in the preceding and 110 μg % in the following decade. The mean value for the women in the first half of this decade was the same as that for women in the last half. Therefore this higher value cannot be related to age within the decade.

Approximately one-third of the women between the ages of 40 and 52 years were going through the menopause. Their mean serum iron value was 9 μg % lower than women in this age range who had not reached the menopause, and 10 μg % lower than those who had passed the menopause. The difference, however, was not significant.

The nutritional and medical significance of the decrease of serum iron with advancing age in healthy women is not apparent. The process of aging may modify those physiological factors which influence the level of serum iron in such a way as to cause this decrease.

Summary. (1) The serum iron and hemoglobin values were determined for 275 healthy women ranging in age from 17 to 86 years. Mean values for all women were 116 ± 33 μg % and 13.2 ± 0.9 g %, respectively. (2) Analysis of variance indicated a difference of serum iron levels among different age groups which was significant at the 1% level. The regression coefficient of serum iron on age was —.343, indicating a decrease of serum iron with advancing age. This decrease was not accompanied by a decrease in hemoglobin values, nor was there a correlation between serum iron and whether the menopause had been reached or passed. (3) There is no apparent nutritional or medical significance to the decrease of serum iron levels with age in healthy women.

Received May 18, 1951. P.S.E.B.M., 1951, v77.

Pressure Pulse Recording from an Isolated Mammalian Heart Preparation Based on a New Principle.* (18891)

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Certain problems of cardiodynamics can be studied profitably by the use of an improved isolated mammalian heart preparation. The mode of relaxation of the ventricle, for example, may hold further information concerning the control of cardiac output, for it is increasingly apparent that the remarkable property of the heart is not only that it can pump so much, but also that it can fill so efficiently. A study of the relaxation process of the ventricle is difficult, however, when large volumes of blood are being pumped by the heart.

In order to study the relaxation process adequately an isolated heart preparation should possess the following attributes: (a) generate ventricular pressure pulses of normal contour, (b) possess easy control of atrial and ventricular systolic and diastolic pressures, (c) pump no blood, *i.e.*, have a fixed ventricular volume, and (d) not deteriorate over a period of many hours. The preparation to be described possesses all these characteristics to a satisfactory extent.

The preparation. Heyman and Kochman (1) apparently introduced the idea of using a donor dog to supply the blood and pressure head for the perfusion of the coronary arteries of the isolated heart. Although their

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* Supported by a grant from the Life Insurance Medical Research Fund.

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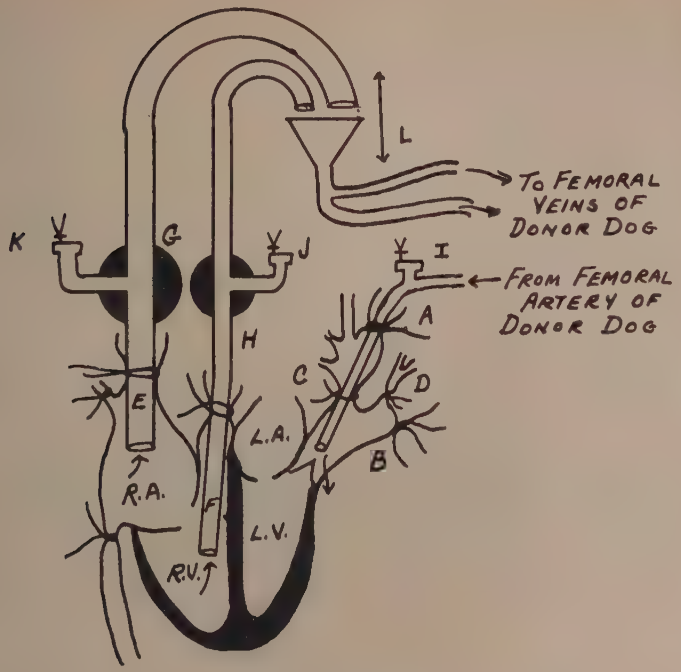
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TO FEMORAL VEINS OF DONOR DOG
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B
R.A.
F
L.V.
R.V.

FIG. 1. Schema used to perfuse the dog heart *in situ*. Explanation in text.

method has not been used extensively it has an advantage over the use of mechanical perfusion systems, namely, that the presence of the intact lungs, liver and kidney of the donor dog can maintain a more normal blood chemistry.

Fig. 1 shows the schema currently used. Blood from the femoral artery of the donor dog enters the ascending aorta via a cannula (A) introduced through a common carotid artery. The aorta is tightly ligatured at the arch (B), as are all head vessels (C) and the left subclavian artery (D). The only pathway for escape of the perfusion blood is through the coronary artery orifices of the isolated heart or through the orifices of the vasa vasorum vessels in a short segment of the ascending aorta and aortic arch.

The coronary venous blood is returned to the donor dog either through a large cannula (E) placed in the superior vena cava, the inferior vena cava and the azygos vein being ligated, or through a cannula (F) placed in the right ventricle by retrograde passage past the pulmonic valve. Manipulation of stopcocks (G) and (H) determines through which channel the coronary venous blood shall return to the donor dog and the manner in which the right ventricle shall contract, *i.e.*, "isometrically" or "isotonically." Optically recording manometers measure the perfusion pressure (I), right ventricular pressure (J), and right atrial pressure (K). The height of the opening of the outflow tubes (L) in relation to each other controls the systolic and diastolic pressure when stopcocks G and H are open.

The consecutive steps in making the preparations are as follows: Two dogs of average size are anesthetized with morphine sulfate and sodium barbital. The chest of the dog whose heart is to be isolated is opened widely by a midsternal incision under adequate artificial respiration. Loose ligatures of strong cotton cord are placed around vessels indicated in Fig. 1. The ligatures passed around the azygos vein and left subclavian artery are tied immediately.

The donor dog is prepared next. Both femoral veins and one femoral artery are exposed with a minimum of trauma. Short metal cannulas of large diameter are tied into one femoral vein and into the femoral artery. The donor dog is then heparinized (4 mg heparin/kg, immediately, and 5 mg each half hour thereafter). A saline filled piece of Tygon tubing 60 cm long and 5 mm I.D. (internal diameter) tipped with a straight brass cannula 15 cm long and 3 mm I.D. is connected to the metal cannula previously placed in the femoral artery. A 100 mm laboratory funnel serves as the receptacle for the blood returning from the recipient heart. A *Y* tube is attached to the funnel and each arm of the tube is fitted with about 40 cm of 5 mm I.D. tubing. After filling with saline, one tube is attached to the metal cannula previously placed in the femoral vein. The other tube is attached to a No. 14 urethral catheter which is introduced well into the inferior vena cava via the other femoral vein. The height of the funnel is adjusted in accordance with the donor dog's venous pressure so that there is the least practical volume of saline in the funnel. The donor dog's animal board is placed on a level about 30 cm below that of the recipient dog's board to facilitate the return of blood to the donor.

The recipient dog is now heparinized in the same manner as the donor dog. The brass perfusion cannula connected to the tubing leading from the donor dog's femoral artery is introduced into the ascending aorta of the recipient dog via a common carotid near its origin and tied in tightly. It is essential that the perfusion line must be free from air bubbles. A large cannula 10 cm long and 1 cm I.D. equipped with a 3-way stopcock and about 40 cm of large tubing (1 cm I.D) is introduced into the right atrium via the superior vena cava and tied in tightly. This cannula and tubing is also filled with saline, but the presence of air bubbles is immaterial. Pinch clamps are used to guard this tubing and the perfusion tubing from emptying during manipulation. A cannula 15 cm long and 4 mm I.D. connected with a large 3-way stopcock and 40 cm of Tygon tubing 5 mm I.D. is filled with saline and the end opposite the cannula fixed in position over the receiving funnel (as is the end of the tubing attached to the cannula in the superior vena cava). This completes the preliminary preparations.

The heart is isolated by quickly performing the following steps in sequence: (1) Tie off the brachiocephalic artery, (2) Tie off the inferior vena cava, (3) Remove pinch clamp from the outflow tubing leading from the right atrium, (4) Tie off the aorta tightly, (5) Remove pinch clamp from the perfusion line, (6) Insert and secure cannula into right ventricle via pulmonary artery, (7) Remove the pinch clamp from the tubing draining the right ventricle. If the above steps have been properly done there is no cardiac standstill or arrhythmia. The coronary venous blood will return in good quantity (25-100 cc/min.) either by way of the right atrium or right ventricle, depending upon the arrangement of the height of the respective outflow tubes and the 3-way stopcocks.

Since the blood cools considerably during its passage through the external circuit, it is advisable to supply heat to the donor dog. Gauze or nylon filters have been used in the receiving funnel, but they probably are unnecessary with adequate heparinization.

Pressure pulse contours. The pressure pulses obtained from this preparation are satisfactory as to contours and pressures. The cardiac chambers may be arranged to beat against either an open or closed outflow system. Segments A, B, C, and D, Fig. 2, show ventricular and atrial pressure pulses recorded from the same animal in various combinations.

Control of pressures. The ease with which intra-cavitary pressures are controlled varies with the combination of opened and closed outflow systems used. When both the atrial and ventricular outflow systems are open the diastolic pressure of both atrium and ventricle can be adjusted independently by varying the relation between the height of the opening of the outflow tubing. When one outflow system is closed the diastolic pressure in that chamber will follow the changes in the dias-

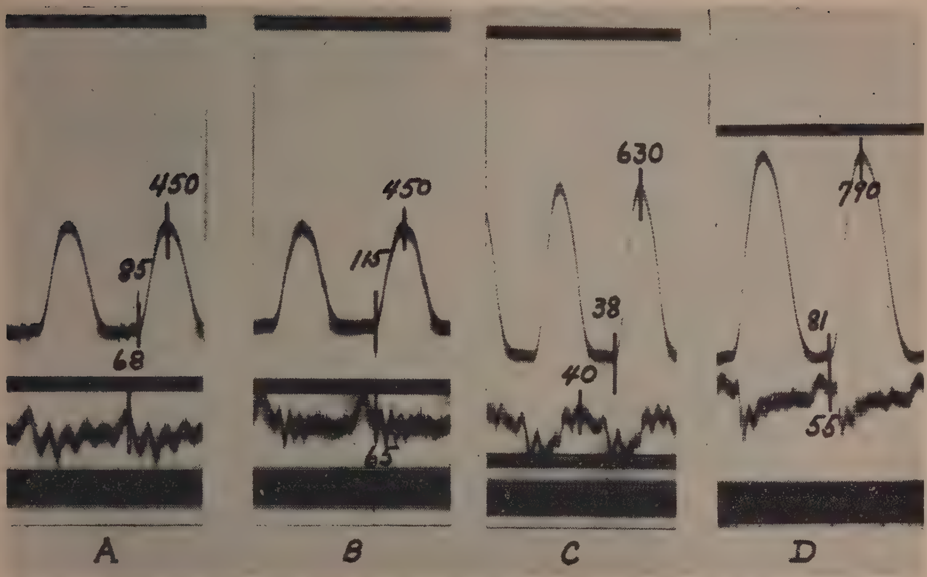

FIG. 2. Right ventricular curves (top) and right atrial curves (bottom) recorded from isolated hearts. A, both ventricle and atrium contracting "isotonically;" B, right atrium contracting "isometrically," and right ventricle "isotonically;" C, right atrium contracting "isotonically" and right ventricle "isometrically;" D, both right ventricle and atrium contracting "isometrically." Calibration in mm saline. Discussion in text. (Note: the terms "isometrically" and "isotonically" are used only to indicate the relative amount of myocardial fiber shortening.)

tolic pressure of the open system. With both outflow systems closed, the pressure in both chambers will increase until failure ensues.

Ventricular isometric contraction. It was hoped that the ventricle could be arranged so that it contained a fixed (and adjustable) volume of blood throughout the cardiac cycle, *i.e.*, a true isometric ventricle. Visual impression indicates that this is impossible in the dog's right ventricle since there is apparent diminution of the size of the ventricle during systole when the right ventricular overflow system is completely closed. This occurs at all pressure levels. At moderate to high pressure levels (above ca 150 saline diastolic) tricuspid regurgitation supervenes, judged by the development of a palpable thrill. Whether or not other functional avenues of escape of blood from the ventricle (Thebesian veins?) exist must be considered. However, the changes in volume of the ventricle during a cycle apparently are slight.

By keeping the ventricular volume relatively fixed during the entire cardiac cycle it has been possible to demonstrate that ventricular relaxation can occur up to the onset of the next systole. Fig. 3, A and B, shows two segments taken from a record in which the heart rate slowed slightly when the systolic pressure increased and systole shortened as the result of injection of 40 μg of epinephrine. The early diastolic portion of the relaxation curve became steeper and the remainder of the diastolic portion of the curve continued to decline until the onset of the next systole. Atrial pressure was less than ventricular throughout diastole (although rising slightly) until late in the atrial systolic phase. Thus, ventricular filling from the atrium must have been negligible. The zero level for both atrium and ventricle was the same.

Viability. As long as an adequate perfusion pressure is maintained these isolated hearts show no tendency to fail. Many experiments have been terminated at the end of 4 or 5 hours with the isolated heart showing a considerable amount of reserve (as tested by increasing the diastolic pressure to abnormal

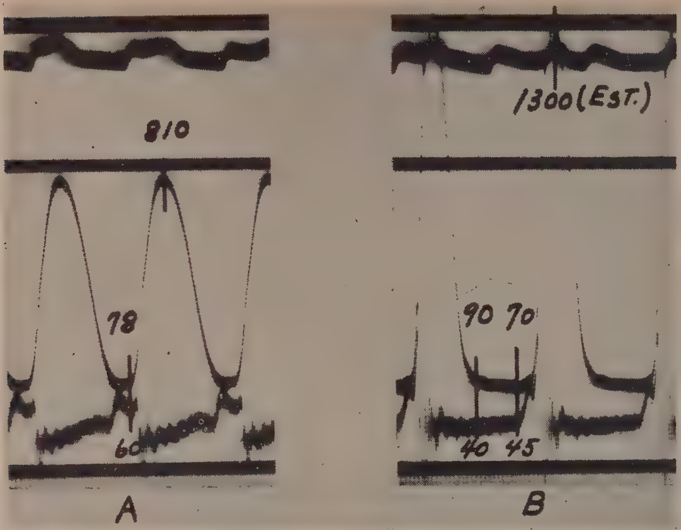

FIG. 3. Right ventricular curves (middle) and right atrial curves (lower) recorded from the isolated heart before (segment A), and after (segment B) an exceedingly small dose of epinephrine. (The top curve is perfusion pressure.) Discussed in text.

levels) without deterioration of the pressure pulses.

Failure of the preparation, when it occurs, is the result of an unexplained default of the donor dog. Preliminary findings indicate that the condition resembles that of irreversible hemorrhagic shock, but the condition is not accounted for on the basis of external blood loss.

Received May 8, 1951. P.S.E.B.M., 1951, v77.

Spectrophotometric Determination of Tromexan in Plasma and Serum.*

(18892)

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The ethyl ester of bis 3,3′ (4-hydroxy coumarinyl) acetate, Tromexan, is available for therapeutic use as an anticoagulant. It is presumed to compete with vit. K in hepatic synthesis of prothrombin. The anticoagulant activity is gauged by the decrement in plasma prothrombin.

The absorption, distribution, destruction, and excretion of Tromexan were studied in part by Pulver and von Kaulla(1). These authors used an indirect colorimetric method of analysis. It is the purpose of this paper to present an accurate and rapid method for the determination of Tromexan levels.

The test. Tromexan is extracted by 2,2,4-trimethylpentane from plasma or serum which has been acidified with hydrochloric acid. The optical density of the resultant solution of Tromexan is determined at a wave length of 310 mμ in a Beckman spectrophotometer. *Reagents.* (1) Concentrated hydrochloric acid (C.P.). (2) 2,2,4-trimethylpen-

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* This work was supported by a grant from Geigy Co.

1. Pulver, R., and von Kaulla, K. N., *Schweiz. med. Wchnschr.*, 1948, v78, 956.

TABLE I. Tromexan Recovered from One Cubic Centimeter of Plasma.

ng Tromexan added	mg Tromexan recovered	% recovered
.01	.0095	95
.01	.009	90
.01	.010	100
.02	.018	90
.02	.020	100
.02	.020	100
.04	.038	95
.04	.037	92.5
.04	.039	97.5
.06	.057	95
.06	.060	100
.06	.058	96.7
.08	.078	97.5
.08	.084	105
.08	.078	97.5
.10	.089	89
.10	.097	97
.10	.102	102
.15	.143	95.5
.15	.143	95.5
.15	.148	98.7
.20	.196	98
.20	.190	95
.20	.200	100
.25	.253	101.2
.25	.240	96
.25	.247	98.8
.30	.296	98.7
.30	.298	99.3
.30	.298	99.3
	Avg recovery—97.2%	S.D. ± 3.5%

ane. *Apparatus.* (1) 30 cc glass stoppered ottles. (2) Shaker. (3) Beckman photo- lectric quartz spectrophotometer with ultra- iolet accessories and matched quartz cuvettes ith 1 cm light path length.

Procedure. One ml of plasma or serum is laced in the glass stoppered bottle. Five ml f 2,2,4-trimethylpentane and 0.1 ml of oncentrated HCl (C.P.) are added. For uantities greater than 0.2 mg of Tromexan er ml of plasma, 10 ml of the organic solvent hould be used. The mixture is shaken for 0 minutes. (In our experiments emulsions ere rarely formed. When they occurred hese emulsions were broken by centrifugation or 10 minutes at approximately 1000 r.p.m.). The supernatant is decanted into a quartz uvette of 1 cm light path length. The optical density is determined at a wave length of 10 mμ. One ml of water (control plasma r serum is preferable) is treated similarly for use as a blank.

Standardization and accuracy of the procedure. Ten mg of Tromexan were dissolved in 100 ml of 2,2,4-trimethylpentane. Amounts of this solution corresponding to 0.005 to 0.2 mg were placed into 30 ml glass stoppered bottles. The solvent was evaporated at room temperature by suction. To each bottle 1 ml of plasma (or serum) was added. The procedure outlined above was then followed. Table I presents the known Tromexan content and the Tromexan content as measured by this method. Over the range studied, an average of 97.2% of the Tromexan was recovered. The standard deviation in these experiments was ±3.5%. The ultraviolet absorption spectra of Tromexan in 2,2,4-trimethylpentane and in 0.1 N NaOH are shown in Fig. 1 and 2. The peak at 310 mμ was chosen for quantitative measurements since materials extracted from normal plasma of humans or dogs do not interfere with the quantitation at this wave length. Standards prepared in accordance with the procedure described show that solutions of Tromexan in 2,2,4-trimethylpentane vary in optical density in accordance with Beer's law (Fig. 3). The dog's plasma used throughout these experiments was obtained by adding 1 cc of

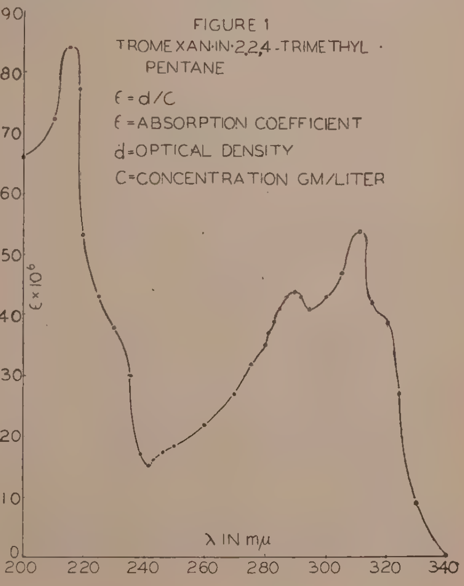


FIGURE 1

TROMEXAN·IN·2,2,4-TRIMETHYL · PENTANE

$\epsilon = d/C$

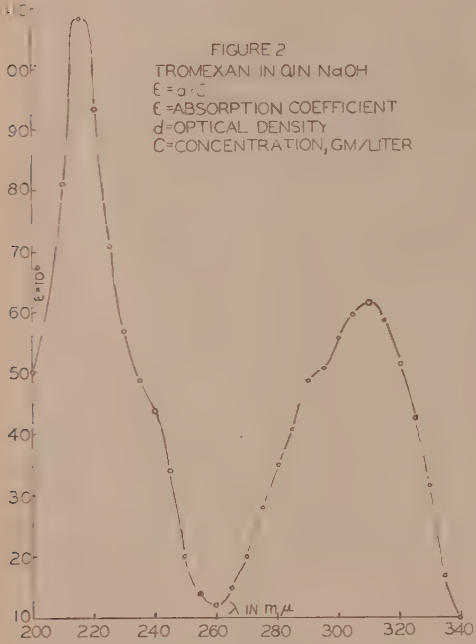
ϵ = ABSORPTION COEFFICIENT

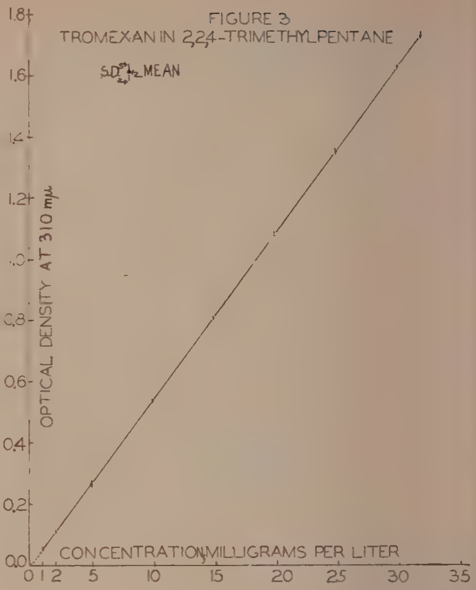
d = OPTICAL DENSITY

C = CONCENTRATION GM/LITER

3.2% sodium citrate solution to every 9 cc of whole blood. Tromexan content determinations may be made with equal accuracy on plasma or serum using the test described above.

Conclusions. (1) Tromexan in amounts of .005 to .2 mg partitions from 1 ml of acidified plasma into 5 ml of 2,2,4-trimethylpentane to the extent of 97.2 ± 3.5%. (2) The optical density of such solutions varies in accordance with Beer's law. (3) On this basis a procedure for estimating plasma and serum concentrations of Tromexan by ultraviolet spectrophotometry was evolved.





Received June 1, 1951. P.S.E.B.M., 1951, v77.

Effect of Lactate on Renal Tubular Transfer of p-Aminohippurate in Man. (18893)

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The presence of sufficient quantities of acetate, pyruvate, or lactate in the ambient fluid of the renal tubule is associated with an increased uptake of p-aminohippurate by the tubular cells *in vitro* and an increased $\mathrm{Tm}_{\mathrm{PAH}}$ in dogs(1,2). The present study was undertaken to determine the effect of parenterally administered lactate on $\mathrm{Tm}_{\mathrm{PAH}}$ in human subjects.

Method. The effect of intravenous administration of lactate on $\mathrm{Tm}_{\mathrm{PAH}}$ was determined for 8 male subjects free of demonstrable renal

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* Present address: U. S. Marine Hospital, Baltimore, Md.

1. Cross, R. J., and Taggart, J. V., *Am. J. Physiol.*, 1950, v161, 181.

2. Mudge, G. H., and Taggart, J. V., *Am. J. Physiol.*, 1950, v161, 191.

TABLE I. Effect of Intravenous Infusion of Na Lactate or Na Bicarbonate on Glomerular Filtration Rates and Tm_{PAH} in Humans.

Subject		Cl_{IN}, cc/min		Tm_{PAH}, mg/min		pH_s at 38°		$(BHCO_3)_s$, meq/l		Plasma lactate, meq/l	
Age	Exp	Control*	Exp†	Control*	Exp†	Control*	Exp†	Control*	Exp†	Control*	Exp†
					Na-lactate administration						
40	A	96.5	115.8	76.8	97.4	7.43	7.47	24.5	29.2	.49	5.95
	B	99.8	111.3	76.2	95.4	7.43	7.49	25.2	28.5	.38	4.50
62	A	63.5	78.8	84	99.6	7.44	7.48	25.6	29.7	.63	4.92
	B	67.5	85.6	84.1	112.1	7.48	7.50	24.6	30	.53	5.80
59	A	91.3	112.4	55.8	104.9	7.48	7.52	27.8	29.8	.50	5.36
	B	74.6	87.6	72.1	89.1	7.47	7.51	27.1	31.1	.45	5.34
51	A	82.7	93.1	52.4	71.2	7.40	7.48	26.7	30.8	.42	5.86
	B	81.3	90.9	55.9	76.4	7.44	7.51	26.8	31.8	.38	5.21
65	A	73.2	99.5	69.6	81.2	—	—	—	—	.45	8.91
	B	75.7	92.5	72.4	79.1	7.45	7.49	24.8	29.2	.50	5.58
63	A	66.1	79.2	72.3	88.2	7.42	7.47	24.1	27.7	.57	5.87
	B	65.2	74.3	72.4	90	7.40	7.45	25.5	28.3	.38	3.44
57	A	76.9	87.1	63.9	85.4	7.42	7.50	26.7	30.3	.62	5.98
	B	79	93.9	70.6	81.2	7.44	7.50	26.7	29.4	.49	3.67
70	A	68.2	79.7	71.5	88.9	7.41	7.46	24.8	27.7	.74	3.18
	B	79	81.2	66.1	81.7	7.42	7.47	22.4	24.8	.62	3.21
Mn		77.53	91.43	69.76	88.86	7.434	7.486	25.55	29.22	.509	5.174
					Na-bicarbonate administration						
51		82.7	88.7	48.5	51.8	7.38	7.46	27.7	34.2	.61	.46
62		66.1	72.6	78.5	83.7	7.44	7.46	26.2	29.6	.65	.54

* Mean of three 12-min urine collection periods before administration of Na lactate or Na bicarbonate.

† Mean of last four 12-min urine collection periods during administration of Na lactate or Na bicarbonate.

disease. Each subject was studied on 2 different occasions at intervals of a week or more. The experimental design was the same for all subjects except as noted. Using the saturation method outlined by Goldring and Chasis(3), 3 control urine collection periods, each of 12 minutes duration, were obtained for the estimation of Tm_{PAH}. During the first 5 minutes of the fourth collection period 100 cc of M r-lactate (100 mM) was injected intravenously. Immediately after this priming dose of M lactate, an intravenous infusion of 200 cc of 0.5 M lactate at a rate of approximately 4.0 cc/min. (2.0 mM/min.) was instituted. Following this fourth period which was 15 minutes in length, 4 more 12-minute collection periods were obtained. In one subject, age 70, only the lactate priming dose was given for the 2 tests. Arterial blood samples for determination of lactate, plasma inulin and PAH concentrations and serum pH (pH_s) and $BHCO_3$ concentrations, $(BHCO_3)_s$, were obtained for every urine collection period except the second control period. To evaluate the relationship of changes in the pH_s and $(BHCO_3)_s$ to the observed increase in Tm_{PAH} 2 subjects were studied during the infusion of 200 cc of 0.45 M sodium bicarbonate following a primer dose of 100 cc of 0.9 M sodium bicarbonate. In 3 subjects the effect of the administration of the hypertonic solution of lactate on the renal blood flow was determined under the same condition of lactate administration that existed for the Tm_{PAH} determinations. Inulin was determined by the method of Harrison(4). PAH determinations, using diluted urine and cadmium sulfate filtrates of plasma, were made by the method of Bratton and Marshall(5). Lactate was determined according to the method of Barker and Summerson(6). The glass electrode method of

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3. Goldring, W., and Chasis, H., *Hypertension and Hypertensive Disease,* The Commonwealth Fund, New York, 1944.

4. Harrison, H. E., PROC. SOC. EXP. BIOL. AND MED., 1942, v49, 111.

5. Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.,* 1939, v128, 537.

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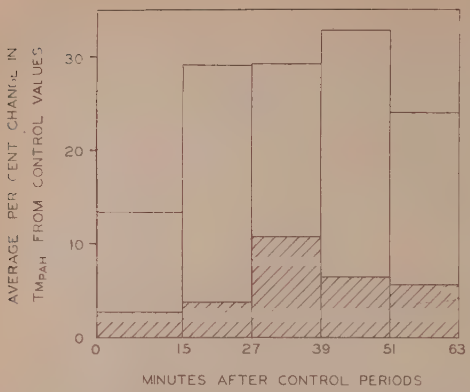

FIG. 1. Height of the clear columns represents the mean % change in Tm_{PAH} of 8 subjects receiving 200 mM of lactate intravenously for 5 consecutive urine collection periods. Height of the hatched columns represents the mean % change in Tm_{PAH} of 2 of 8 subjects receiving 180 mM of bicarbonate intravenously.

Dill, Daly, and Forbes(7) was used for the measurements of pH_s, and total CO_2 content of the blood was determined by the micromethod of Shock and Hastings(8). $(BHCO_3)_s$ values were computed by use of a nomogram(9).

Results. Following the injection of the lactate primer and during the lactate infusion there occurred a significant increase in C_{IN}, Tm_{PAH}, pH_s, $(BHCO_3)_s$, and blood lactate concentration (Table I). Increases were observed in all 16 experiments on the 8 subjects. The average inulin clearance increased from 77.53 to 91.43 cc/min. ($t = 84$, $P < .01$) and the average Tm_{PAH} increased from 69.76 mg per min. to 88.86 mg/mm ($t = 8.0$, $P < .01$). Comparable t values for pH_s, $(BHCO_3)_s$, and blood lactate concentration changes were 8.4, 13.2, and 11.2, respectively, all of which yield $P < .01$. The maximum mean per cent increase in Tm_{PAH} for the entire group of subjects following the injection of lactate was 33% and occurred during the urine collection period 39 to 51 minutes following the beginning of lactate administration (Fig. 1).

The pH_s and $(BHCO_3)_s$ displacements produced by the intravenous administration of sodium bicarbonate in 2 subjects (ages 51 and 62) were the same as those induced by lactate, but the administration of bicarbonate effected average increases in Tm_{PAH} of only 6.6 and 6.7%, respectively (Table I, Fig. 1). The intravenous administration of sodium bicarbonate in amounts of 10 and 14 g did not cause an elevation in the blood lactate levels contrary to what might be expected from other reports (Table I)(10,11,12).

In the 3 subjects studied, the average increase in renal blood flow was 11.2% of control values, and there was no appreciable change in the filtration fraction (Table II).

Discussion. The increase in Tm_{PAH} resulting from the lactate administration is considered to be predominantly a result of an increased rate of tubular cell excretion of PAH. The possibility of the observed changes in PAH excretion being due to an increased pH_s and/or $(BHCO_3)_s$ is remote in view of the failure to produce changes in Tm_{PAH} with increased pH_s and $(BHCO_3)_s$ by the administration of sodium bicarbonate comparable to those produced by lactate administration.

The possibility that the observed increases in Tm_{PAH} resulted from a perfusion of an increased number of nephrons is unlikely. The

TABLE II. Effect of Intravenous Infusion of Na Lactate on C_{IN} and C_{PAH}.

Subject Age*	Inulin clearance, cc/min Control†	Inulin clearance, cc/min Exp‡	PAH clearance, cc/min Control†	PAH clearance, cc/min Exp‡	Lactate, meq/l Control†	Lactate, meq/l Exp‡
51	86.4	100	478.9	537.7	.57	4.97
59	82	91	436.5	466.8	.90	5.81
62	75.9	84	369.4	424.1	.99	4.96
Mean	81.43	91.67	428.27	476.20	.820	5.247

* Same subjects as shown in Table I. Identified by age.

† Mean of three 12-min urine collection periods before administration of Na lactate.

‡ Mean of last four 12-min urine collection periods during the administration of Na lactate.

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8. Shock, N. W., and Hastings, A. B., *J. Biol. Chem.*, 1934, v104, 565.

9. Hastings, A. B., and Shock, N. W., *J. Biol. Chem.*, 1934, v104, 575.

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in vitro studies of Cross and Taggart(1) have demonstrated that the rate of PAH accumulation by thin slices of rabbit kidney cortex is accelerated by the presence of acetate, pyruvate or lactate in the suspending medium. Under conditions of greatly decreased renal vascular resistance associated with increases in renal blood flow of 75 to 100 percent there is a small and variable change in Tm_{PAH} (3,13). The increases in renal blood flow observed under the present experimental conditions were between 10 and 15%. Even if this increase were indicative of a perfusion of previously ischemic nephrons, it would not account for the increases in Tm_{PAH} observed.

Summary. The intravenous administration of sodium lactate causes a statistically significant increase in Tm_{PAH} in man. The average maximum increase amounted to 30% of the preinjection levels, and the effect was observed in each of 8 subjects tested twice. Neither the observed increases in pH_s and $(BHCO_3)$ nor the changes in renal blood flow appear to bear a causal relationship to the increases in Tm_{PAH} produced.

The technical assistance of Mrs. Elsie Beard, Miss Margaret McCollum, Mr. Arthur Dinan, and Mr. W. Irving Jones is gratefully acknowledged.

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Received June 18, 1951. P.S.E.B.M., 1951, v77.

Evaluation of Segmental Gastric Resection for Peptic Ulcer.* (18894)

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For many years surgeons have done a three-quarter gastric resection for peptic ulcer in which the entire gastrin-containing area of the stomach along with a large segment of the acid-secreting area is excised utilizing the Billroth I or Billroth II technics. In order to overcome some of the limitations of these methods, it was proposed(1) to reverse the usual procedure of gastric resection by leaving the antrum intact, excising a larger fragment of the acid-secreting area, and leaving not more than 10% nor less than 5% of the total gastric area in the fundic segment. Diminution of acid secretion is then assured, and optimal conditions afforded for neutralization and digestion by bile and pancreatic juice. Moreover, it was hoped that segmental gastric resection might obviate some of the problems of the "dumping syndrome" observed rather commonly following the conventional types of gastric resection.

Berger(2) has mapped out the parietal cell distribution on 8 normal stomachs. He pointed out that there are virtually none of these cells in the antral area, and that there are 50% as many parietal cells in the fundic and cardiac portions of the stomach as in the corpus. Ferguson(3) showed in a dog with the stomach divided into 4 distinct pouches that the upper pouch, representing 19% of the stomach by weight, secreted half as much acid per g of stomach on histamine stimulation as the middle area of the stomach representing 60% of the stomach by weight. This finding correlates well with Berger's parietal cell counts. The present study was undertaken to ascertain how much of the acid-secreting area of the stomach needs to be removed to protect against ulcer formation when the gastric antrum is allowed to remain intact.

Present study. Six series of dogs were studied.

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* This study was supported by the following grants: 1. The United States Public Health Service (Ulcer Project), Contract RG1026-C4; 2. The Augustus L. Searle Fund; 3. The Austen S. Cargill Fund; and 4. The Graduate School of the University of Minnesota.

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TABLE I. Assessment of Protection of Segmental Gastric Resection in Dogs Against Histamine-in-Beeswax Provoked Ulcer. (Daily dose 30 mg histamine base.)

Operation	No. dogs	Avg No. Histamine injec	Avg % by wt of fundic pouch: Estimated at operation, %	Avg % by wt of fundic pouch: Weighed at autopsy, %	Avg % by wt of acid secreting area excised, %	Ulcers		Erosions	
						No.	%	No.	%
Series 1									
Resection of entire upper stomach; antrum anastomosed to esophagus	4	45	0	5	94	0	0	0	0
Series 2									
Resection of corpus; fundic-antral anastomosis	10	47	10	20	74	0	0	0	0
Series 3									
Resection of corpus; fundic-antral anastomosis; pyloroplasty	6	45	10	21	72	0	0	0	0
Series 4									
Resection of corpus; fundic-antral anastomosis; pyloroplasty	6	45	25	31	63	1	16.6	1	16.6
Series 5									
Resection of corpus; fundic-antral anastomosis; pyloroplasty, vagotomy	6	42	25	33	60	3	50	0	0
Series 6									
Controls—no operation. Dogs received same amount of histamine	6	29	—	—	0	6	100	0	0

Series 1. Excision of the entire acid-secreting area of the stomach with esophagoantral anastomosis (4 dogs).

Series 2. Excision of the corpus leaving 10% of the stomach by weight as a fundic pouch with fundic-antral anastomosis (10 dogs).

Series 3. Same as Series 2 with a pyloroplasty added to hasten gastric emptying (6 dogs).

Series 4. Excision of the corpus leaving 25% by weight of the stomach as a residual fundic pouch with fundic-antral anastomosis (6 dogs).

Series 5. Same as Series 4 with transthoracic vagotomy added (6 dogs).

Series 6. (Controls). Six dogs, not operated upon, receiving the same daily dose of histamine-in-beeswax, were employed as controls to test the effectiveness of the histamine-in-beeswax preparation in the production of peptic ulcer.

All operations were carried out on healthy mongrel dogs weighing from 24 to 50 lb. Anastomosis was accomplished by the closed method, great care being taken to preserve the fibers of the vagus nerves to the antral segment along the lesser curvature in the first 4 series of animals. The pyloroplasty in Series 3, 4 and 5 was made as an open, linear pylorotomy with transverse closure of the Heinecke-Mikulicz type. It is difficult to determine exactly the area of the antrum from the external appearance of the stomach. In order to leave only antral mucosa in the lower pouch, the distal line of resection transected the stomach from a point just above the incisura on the lesser curvature almost perpendicularly downward to the greater curvature. To obtain a 25% fundic pouch, the proximal line of resection was extended from between the second and third short gastric vessels on the greater curvature to a point 2 to 2.5 cm below the esophago-gastric junction on the lesser curvature. To obtain a 10% fundic pouch only a narrow strip of stomach was left below the esophago-gastric junction. Patterns of the extent of resection were determined beforehand in preliminary trials on sacrificed dogs. The actual correlation of the

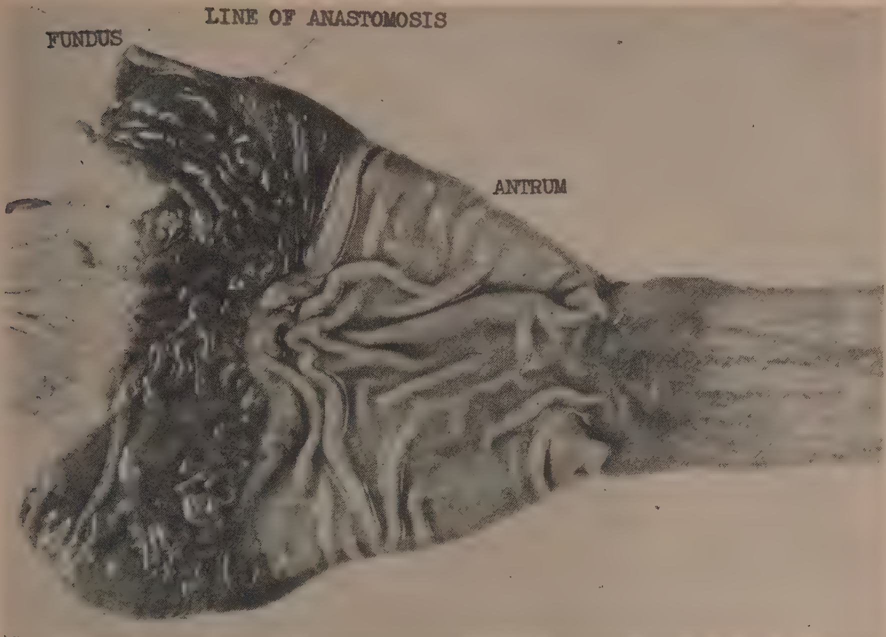

FIG. 1. 10% fundic pouch. Note small triangle of acid-secreting mucosa along the greater curvature on the antral side of the anastomosis.

extent of the excision in the various series had to await the ultimate determination of the weight of the residual gastric fragments when the dogs were sacrificed on the conclusion of the experiments.

Gastric emptying times were done by the method outlined by Lillehei *et al.*(4), and the completeness of the vagotomies in dogs in Series 5 was tested by insulin gastric analysis, using 3 units of insulin per 10 lb of body weight.

All dogs were allowed to recover for at least 30 days postoperatively before starting the daily dose of 30 mg of histamine base in beeswax administered intramuscularly according to the method described by Hay and associates(5). Injections were continued for a period of 45 days unless the animal died of the complications of peptic ulcer. The effectiveness of the histamine preparation in stimulating gastric secretion was tested at periodic intervals throughout the course of the experiment in Heidenhain pouch dogs.

Results. The results of this study are outlined in Table I. The discrepancy noted in the weights of the residual fundic pouch between the predicted values estimated at surgery, on the basis of earlier determinations on sacrificed dogs, and the weights found ultimately on completion of the experiments is explained by hypertrophy of the residual stomach pouch. Moreover, in spite of the care taken at the time of surgery, there occasionally remained a small triangular area of acid-secreting mucosa attached to the antral pouch which had not been excised at the time of the segmental resection (Fig. 1). No essential difference in gastric emptying time was noted in these experiments whether or not a pyloroplasty or a vagotomy was done in addition to the gastric resection.

In the dogs developing gastric ulcers in Series 4 and 5, 2 of the ulcers were at the esophago-gastric junction, and 2 were at the line of anastomosis between fundus and antrum.

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Comments. The present study indicates that an extensive segmental gastric resection is an adequate operation for peptic ulcer if only 10% of the stomach by weight is left as the residual fundic pouch. However, when 25% or more of the stomach is left intact as a fundic pouch, this operative procedure fails to afford protection against the histamine-in-beeswax provoked ulcer. This latter finding is not surprising since 25% of the stomach is the amount found to make a suitable residual pouch to protect against recurrent ulcer in the conventional 75% gastric resection in which the antrum is also removed. Ferguson(3) has shown that vagotomy decreases histamine stimulated secretion by 68% in total gastric pouches, as contrasted with the observed secretion values from these same pouches before denervation. Nevertheless, a complemental vagotomy added to segmental resection when a 25% fundic pouch remains, fails to protect against the histamine-in-beeswax provoked ulcer (Series 5).

Summary. (1) An extensive segmental gastric resection in which 74% of the acid secreting portion of the stomach by weight is excised, leaving 10% of the entire stomach as the upper fundic pouch anastomosed to the antrum will protect against the histamine-in-beeswax provoked ulcer in the dog. (2) A segmental resection in which 25% of the stomach by weight is left as the fundic pouch will not protect against the histamine-in-beeswax provoked ulcer, whether or not a complemental vagotomy is done.

Received June 19, 1951. P.S.E.B.M., 1951, v77.

Phosphocreatine in Red and White Muscle. (18895)

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The striated muscle of the turkey was reported by Krishnan and Nelson(1) to be a superior source of adenosine triphosphate as compared with that of a number of mammals. These same investigators noted that the white breast muscle provided a greater yield of this high energy phosphate than did the red muscle of the leg. Lohman(2) demonstrated that the pyrophosphate content of trichloracetic acid extracts of white muscle of the rabbit was approximately 1.5 times that of the red muscle. These findings suggested the possibility that a difference might also be found in the phosphagen (phosphocreatine) content of these muscles which might further reveal some relationship of this constituent to muscle activity.

An experiment was designed to ascertain relative levels of the creatine phosphate in the leg and breast muscles of the common fowl, and furthermore to compare these results with those obtained in similar muscles of "flying" birds, *i.e.* the pigeon.

Experimental. The analyses were made on samples of the anterior muscles of the thigh and the pectoral muscle of 6 White Leghorn pullets* and 6 pigeons of indefinite breed. All animals appeared to be in good flesh and health. No discrimination as to stage of the reproductive cycle was made in selecting the animals. Uniformity of experimental animals was of limited importance since the comparisons were made between the red and white muscles of each animal separately. Each pair of determinations were completed simultaneously and under identical conditions. The animals were anesthetized by the slow introduction of sodium pentobarbital intravenously, and were bled immediately prior to the removal of the muscle samples. The samples were taken alternately first from the breast or

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* The birds were furnished by Dr. J. H. Bruckner, Poultry Department, Cornell University Agriculture Experimental Station.

TABLE I. Phosphate Content in Bird Muscle.

Animal	Phosphorus, mg/100 g of muscle Ortho Breast	 Leg	 True inorganic Breast	 Leg	 Phosphagen Breast	 Leg
Fowl 1	91.2	93.3	20.7	61.4	70.5	31.9
2	86.3	92.5	30	72.5	56.3	20.1
3	97	81.4	53.3	66.8	43.7	14.6
4	86.8	77.3	13.3	51.9	73.5	25.4
5	83.8	76	16.2	42.2	67.6	33.8
6	99.4	81.9	12.4	34.6	87	47.3
Avg	90.8	83.7	24.3	54.9	66.4	28.9
Pigeon 1	80	92	46.3	54.1	33.7	37.9
2	69.8	91.3	43.3	60	26.5	31.3
3	87.7	91.8	54.1	47.7	33.6	44.1
4	83.6	93.2	50.5	60	33.1	33.2
5	95.7	115.1	43.7	54.7	52	60.4
6	87.2	86.4	47.3	36.5	39.9	49.9
Avg	84	95	47.5	52.2	36.5	42.8

the leg in the different birds.

The procedure of Fiske and Subbarow(3) as adapted by LePage(4) was followed for the separation of phosphocreatine. Phosphorus estimations were made colorimetrically using the ferrous sulfate method(5).

Table I indicates that the white muscle of the fowl breast contained over twice as much creatine phosphate as the red muscle of the leg. In contrast to these results, the creatine phosphate levels in the red breast muscle of the pigeon approximated those of the leg.

A comparison of red and white muscle was also made in single instances in the turkey and the turtle. Again the white muscle in both animals contained higher levels of the phosphagen as compared to that found in the red muscle in both species.

Discussion. The results obtained here were secured over a period of several months. Seasonal variation might explain the differences observed within a species, particularly with regard to the total phosphates. Wang(6), who reported on the muscles of a large number of rabbits, standardized for race and diet, confirmed the findings of other workers, that the phosphagen variations are real.

The phosphocreatine data presented here would indicate that the phosphagen reserve bears an inverse relationship to the low myoglobin level in white muscle and high content in red muscle reported by Bach(7). The white breast muscle appears to be an adaptation of some birds to meet their needs of bursts of energy for non-sustained flight. This is the antithesis of red smooth muscle where energy needs are fairly constant. Walaas and Walaas(8) reported the creatine phosphate content of smooth uterine muscle as being 1/50 the amount found in skeletal muscle, *i.e.* 1-2 mg %. Glycolysis in this case served by the abundant supply of myoglobin could satisfy the uniform needs for energy without a large reservoir of phosphagen.

The high white-muscle phosphagen levels described in this paper are consistent with Krishnan and Nelson's observation of a higher adenosine triphosphate content in white muscle than in the red muscle. Combined, these facts are complimentary to the explanation offered above for phosphagen reserve and intermittent but high demands for energy.

Conclusion. The creatine phosphate content of the white breast muscle of the fowl was found to be over twice as great as that of the red leg muscle. In the "flying" bird, the

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pigeon, the red breast muscle and the leg muscle contained approximately the same levels of this high energy phosphate.

Received June 20, 1951. P.S.E.B.M., 1951, v77.

The Effect of BAL on Distribution and Metabolism of P^{32} and Sr^{90}. (18896)

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The administration of 2,3-dimercaptopropanol (British anti-lewisite or "BAL") has been reported to be of value in the treatment of poisoning from arsenic(1), mercury(2), and other heavy metals(3). Certain of the heavy metals(3) that produce their toxic effects by combining with —SH groups in the tissues thereby obstructing the processes of oxidation and reduction essential to tissue economy. Due to the high stability of BAL-heavy metal compounds, BAL has been regarded as a useful agent for preventing or reversing tissue —SH combinations with heavy metals and for removing heavy metals deposited in bone. The use of BAL for the removal of internally-deposited radioactive isotopes has been suggested and applied in a few instances with various results(4-6). It was the purpose of this investigation to examine the effect of administration of BAL on the retention and distribution of P^{32} and Sr^{90}.

The animals chosen for the study were C_3H male mice between 5 and 6 months of age. A total of 50 animals was divided into 2 groups of 25 each—one group for injection with P^{32} and the other for Sr^{90} injection.

A solution of BAL in peanut oil, at a concentration of 20 mg per ml, was injected daily into alternate thigh muscles of 15 mice in each group. The daily dose was 0.05 ml or 1 mg BAL per mouse(7). The animals were treated with BAL in this fashion for 3 days prior to injection with radioactive tracer and also after injection in the case of the animals which were retained for more than one day before sacrifice. The remaining 10 mice in each group were injected only with radioactive tracer and sacrificed at intervals as controls for the BAL-treated animals.

One group of 15 BAL-treated and 10 control mice was injected with carrier-free P^{32} at a dose level of 0.1 μc per g. The other group of BAL-treated and control mice was injected with carrier-free Sr^{90} at the same dose level (0.1 μc per g). Injections of both isotopes were made intraperitoneally.

Five animals of each group were sacrificed at each of the following intervals after injection: 0.5, 1, 24, 48, and 120 hours. Of the 5 animals sacrificed per group per interval, 3 were BAL-treated and 2 were controls. The pelt, spleen, liver, kidneys, soft tissue, femur, and residual carcass of each mouse were analyzed separately for their content of P^{32} or Sr^{90}. All measurements were made on the standard Geiger-Mueller end-window counter and compared with standards prepared from the original injection solutions.

In as much as the purpose of the investigation was to determine whether there was any alteration in the normal metabolic pattern, the total retention was of interest, and Fig. 1 presents a graphic plot of per cent retention of

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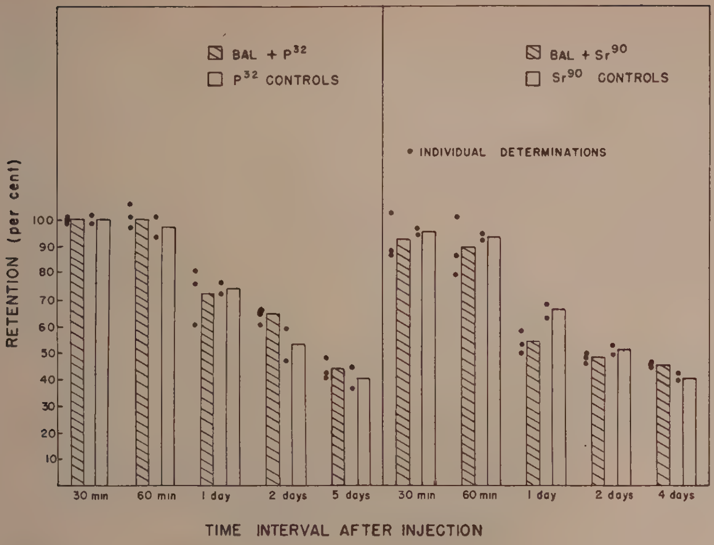

FIG. 1. % retention of P^{32} and Sr^{90} in BAL-treated and control mice.

BAL-treated and control animals. The data indicate that under the conditions of BAL administration there was no effect on retention of P^{32} or Sr^{90} in the treated animals as compared with the normal controls.

Though gross retention was not altered by BAL administration it was of interest to examine the isotopic content of the various organs where differences might appear that were not noticed in the overall retention picture. In Table I is presented the distribution of the radioactive materials as per cent of the

TABLE I. Average % Injected Dose per Organ.

Interval ⟶	30 min		60 min		1 day		2 days		5 days	
Tissue	BAL-treated	Controls	BAL-treated	Controls	BAL-treated	Controls	BAL-treated	Controls	BAL-treated	Controls
				P^{32}						
Pelt	4.59	7.48	5.13	6.61	3.75	4.29	3.56	3.52	2.30	2.40
Spleen	1.15	.78	1.01	1.27	.76	.64	.60	.52	.29	.28
Liver	16.41	13.13	16.46	14.96	6.29	7.46	4.68	4.29	2.69	2.64
Kidneys	3.05	3.57	2.57	3.39	1.31	1.39	1.06	1.06	.66	.72
Soft tissue*	24.28	22	22.83	19.64	13.82	16.67	12.48	11.13	6.90	5.85
Femur	.31	.62	.47	.62	.66	.67	.65	.51	.61	.51
Residual carcass	50.33	55.24	53.03	50.98	46.26	43.19	41.07	32.79	30.45	28.04
Total tissue recovery	100.12	102.87	101.50	97.47	72.85	74.31	64.10	53.82	43.90	40.44
				Sr^{90}						
Interval ⟶	30 min		60 min		1 day		2 days		4 days	
Pelt	8.39	9.20	5.34	4.86	.43	.42	.19	.40	.15	.10
Spleen	.24	.21	.08	.08	.02	.01	.01	.02	.01	.01
Liver	2.63	3.38	1.46	1.91	.13	.17	.12	.23	.06	.06
Kidneys	1.06	1.12	.63	.81	.05	.05	.03	.06	.02	.01
Soft tissue*	12.21	11.85	11.96	11.93	3.17	2.28	1.32	1.68	.72	.52
Femur	1.40	1.56	1.94	1.74	1.79	2.31	1.80	1.83	1.79	1.51
Residual carcass	66.93	68.21	67.90	69.77	48.45	62.92	44.74	47.37	42.82	38.70
Total tissue recovery	92.86	95.53	89.31	91.10	54.04	58.16	48.21	51.59	45.57	40.91

* Visceral organs plus remaining soft tissue.

injected dose per organ. Of the tissues examined, the only instance of a major deviation from control values was in the femurs of animals injected with P^{32}. In this case, the P^{32} content of the femurs of animals sacrificed 30 minutes after injection was only about half that of the controls. The difference was reflected in generally higher values in some of the soft tissues. After 1 hour this difference was reduced considerably and disappeared entirely in mice sacrificed after 1 day. The difference in femur retention of P^{32} at 30 minutes is considered especially significant since the reproducibility of bone values from animal to animal is good. The data further indicate that in the case of Sr^{90} BAL has no effect on either the rate of uptake, distribution, or total retention.

These results indicate that although the intramuscular administration of BAL in mice receiving P^{32} intraperitoneally results in a reduced rate of deposition of the isotope in bone, the differences are obliterated within 24 hours. No differences in the total retention of P^{32}, as compared with controls not treated with BAL, were observed. It would appear, therefore, that the use of BAL as an agent to accelerate elimination of either P^{32} or Sr^{90} from the animal body is not practicable, at least under the conditions of this experiment.

Summary. The effectiveness of 2,3-dimercaptopropanol (British anti-lewisite) in increasing the elimination of injected P^{32} and Sr^{90} in mice was investigated. It is concluded that BAL is essentially without effect although, in the case of P^{32}, the rate of uptake in bone appears to be reduced during the first hour. No such differences could be demonstrated after 24 hours.

Received June 22, 1951. P.S.E.B.M., 1951, v77.

Anaphylactic Hypersensitivity Induced in Rabbits to Foreign Proteins in Freund's Adjuvant.* (18897)

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The method of enhancing the sensitivity of animals to foreign proteins by the incorporation of the antigen in Freund's adjuvant containing *M. tuberculosis* is well known. Although the originators of the method(1) did not believe that the sensitivity to the proteins was more than quantitatively different from that induced by the proteins used alone, the impression has persisted(2,3) that the state of hypersensitivity induced by the foreign proteins in Freund's adjuvant is of the tuberculin type. It may be that this impression dates from the work preceding the description of the adjuvant(4-6), in which it was held that, in tuberculous guinea pigs, the state of hypersensitivity induced to foreign proteins was of the tuberculin type. However, this interpretation was by no means established as fact(7).

The possibility that one might alter the type of hypersensitive reaction to a given foreign protein appeared to be of considerable

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* Assisted by a Grant-in-aid from the National Research Council of Canada.

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importance in considering the relationship between the state of hypersensitivity and the cardiac valvulitis, necrotizing arteritis and glomerulonephritis that can be induced in rabbits by massive injections of horse serum (8-11) or bovine gamma globulin(12-14). Short term experiments employing 2 injections of horse serum or bovine gamma globulin incorporated in Freund's adjuvant containing *M. tuberculosis*(15) indicated that lesions of the heart, arteries and kidneys could be produced in the animals and that the skin sensitivity that developed was of the usual Arthus or anaphylactic type. It was considered desirable to make further observations on animals treated for a longer period of time and to extend them to include observations on the skin reactions to tuberculin and on the possibility of passive transfer of the sensitivity by serum.

Materials and methods. Two groups each consisting of 7 albino rabbits were given intramuscular doses of 0.1 g of lyophilized horse serum protein or bovine gamma globulin in Freund's adjuvant at weekly intervals for 18 weeks. Two further injections were given 8, 16 and 17 weeks after the last injection. Thus the animals received 21 injections in all over a period of 35 weeks. One week after the last injection the 7 surviving animals were bled and skin tested to graded dilutions of bovine gamma globulin or horse serum. The same animals were skin tested with varying amounts of purified protein derivatives (P.P.D.). Some of the animals have died since these tests were made and others are being kept alive to observe the effect of the injected substances over longer periods of time.

The Freund's adjuvant used in these experiments contained a final concentration of 0.6 g of dried horse serum per 5 cc of the mixture or 1.0 g of bovine gamma globulin per 4 cc. These antigens were first suspended in 2 parts of saline in a mortar. This viscid mixture was then combined with 1 part of Falba‡ in the mortar. Following this, 2 parts of Bayol F¶ containing 2.5 mg of killed and dried *M. tuberculosis* per cc, were added to the previous constituents and the whole emulsified in a Waring Blendor. Each injection consisted of 0.4 cc of the adjuvant-globulin mixture and 0.8 cc of the adjuvant-horse serum mixture.

Intradermal tests were made to saline and graded dilutions of horse serum or 10% bovine gamma globulin in the following dilutions: 1/1, 1/10, 1/100, 1/1000, and to P.P.D. in quantities of .0025 mg, .005 mg, and .01 mg as well as to the diluent used to dissolve the P.P.D. The reactions were read at one hour, 24, 48, 72, 96 and 160 hours by measuring the maximum and minimum diameters of the resulting oedema, redness, haemorrhage or necrosis. From these the average diameters of the phases of the reactions were calculated for each individual reading and the results tabulated. Since the results seen in the individual animals did not differ qualitatively from one another whether they received horse serum or bovine gamma globulin, all the average diameters were combined, tabulated in Table I and represented graphically in Fig. 1. The height of the oedema was roughly proportional to its average diameter but the former is so difficult to measure accurately that the numerical data concerning height were not considered suitable for analysis.

Serum from the sensitized rabbits was injected intraperitoneally into guinea pigs in quantities of 2.0-2.5 cc. After 24-30 hours an intravenous shocking dose of 0.2 cc of the appropriate antigen was given.

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‡ Marketed by Pfaltz & Bauer, New York.

¶ Obtained through the courtesy of Stanco, N. Y.

TABLE I. Skin Tests. Average diameter in mm. Avg of all animals tested (7).

Antigen and dilution		Hr after test dose 1	24	48	72	96	160
Saline	0	7.21	0	0	0	0	0
Serum 1/1	R*	0	34.9	35.3	32.8	27.2	19.5
	O	14.02	34.9	35.3	35.1	34.2	24.6
	H	0	8.57	6.6	3.2	.3	0
	N	0	13.07	12.1	10.7	11.9	11.5
1/10	R	0	25.14	18.2	14.6	10.5	7.2
	O	10.62	25.14	19.3	13.9	15	9.4
	H	0	14.5	2.2	0	0	0
	N	0	8.5	7.7	7.14	6.1	5.1
1/100	R	0	14.78	12.2	9.6	5.4	.85
	O	10.78	14.78	12.2	8.9	6.1	.85
	H	0	6.5	1.1	2.3	.9	0
	N	0	.85	1.07	.14	0	.3
1/1000	R	0	6.28	4	1.1	0	0
	O	9	3.2	.8	1.1	0	0
	H	0	0	0	0	0	0
	N	0	0	0	0	0	0
P.P.D. diluent	R	0	2.14	.6	0	0	0
	O	7.28	.72	0	0	0	0
	H	0	0	0	0	0	0
	N	0	0	0	0	0	0
P.P.D. .0025 mg	R	0	5.2	6.9	7	7.1	7.5
	O	7.9	0	1.8	8.8	5.5	5.3
	H	0	0	0	0	0	0
	N	0	0	0	0	0	0
.005 "	R	0	7.92	10.6	11.8	9.6	8.9
	O	11.35	0	5.8	9	9.6	8.9
	H	0	0	0	0	0	0
	N	0	0	0	0	0	0
.01 "	R	0	8.3	11.6	12.9	13.1	10.5
	O	12.35	0	3.6	9.9	11.5	10.5
	H	0	0	0	0	0	0
	N	0	0	0	0	0	0

* R—redness; O—oedema; H—haemorrhage; N—necrosis.

The precipitin titers of the rabbit sera were determined by finding the optimal ratio(16).

Results. From Table I and Fig. 1 it may be seen that the reactions occurring at one hour to serum are very little different from those obtained with the saline injection and those to P.P.D. differed little from that produced by the diluent. Thus the one hour reading may be considered to be a nonspecific one. The slight difference in size of the reactions resulting from the injection of the foreign protein and P.P.D. solutions may be merely a matter of the osmotic pressures of the solutions containing protein preventing the rapid diffusion of the solutions into the tissues. It is also evident from Table I and Fig. 1 that the reactions to serum reached their maximum size in 24 hours and then began to recede. Only with undiluted serum where necrosis of tissue was extensive, did the reactions tend to persist near their original intensity. With the more dilute solutions, a rapid recession occurred. The persistence of the reactions to undiluted serum may therefore be considered to be a result of the marked necrosis of tissue. The fundamental difference between the reactions to the serum and to the P.P.D. may be seen in Table I and in Fig. 1 where it was evident that even in the absence of necrosis of tissue the reactions to P.P.D. tended to be delayed in type and reached their maximum in 72-96 hours and were persistent at almost their maximum intensity until the 7th day. It was therefore concluded that while the tuberculin type of sensitivity did develop in these animals to tuberculin as a result of the injection of whole *M. tuberculosis*

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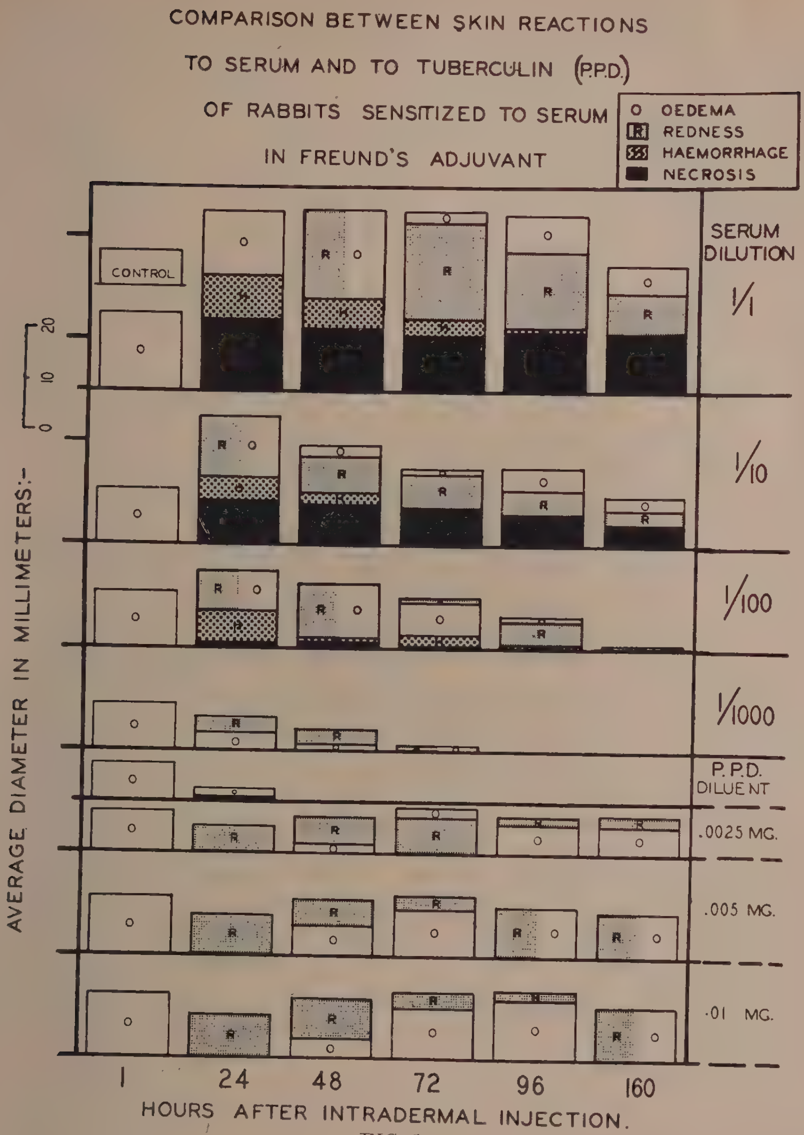

FIG. 1.

a constituent of the Freund's adjuvant, the pe of sensitivity that developed to the serum hile incorporated in this vehicle, was of the pical anaphylactic or Arthus type. While is is true of the reactions in rabbits the pos-bility that the type of skin sensitivity may altered in guinea pigs by use of the ad-vant cannot be excluded.

Further evidence that the sensitivity to the injected serum was of the anaphylactic type was the demonstration of circulating precipitins in the serum of the treated rabbits shown in Tables II and III. In these tables it is also shown that the sensitivity could be passively transferred to guinea pigs in that the majority of the animals manifested fatal

TABLE II. Passive Transfer of Anaphylactic Sensitivity to Guinea Pigs Using Anti-Bovine Gamma Globulin Rabbit Serum.

Rabbit No.	Optimal ratio	No. of guinea pigs sensitized	Anaphylactic shock			
			Fatal	Severe	Mild	None
I-8	1/128	5	5	0	0	0
I-11	1/64	5	5	0	0	0
I-18	1/128	5	4	0	0	1
I-10	1/64	5	2	1	1	1
H-72	1/128	5	3	0	0	2
P-90	Normal Control Serum	5	0	0	0	5

TABLE III. Passive Transfer of Anaphylactic Sensitivity to Guinea Pigs Using Anti-Horse Rabbit Serum.

Rabbit No.	Optimal ratio	No. of guinea pigs sensitized	Anaphylactic shock		
			Fatal	Severe	None
I-7	1/12	7	5	1	1
I-3	1/16	6	4	0	2
I-17	1/16	6	6	0	0
I-20	1/16	6	5	0	1
H-71	1/16	6	5	0	1
L-22 L-65	Normal Rabbit Serum	7	0	0	7

or severe anaphylactic shock when challenged with an appropriate intravenous injection of the antigen.

Summary and conclusions. By the use of 3 criteria, namely: the presence of circulating antibodies in the serum; the mode of development of the skin reactions to intradermal injections of antigen, and the passive transfer of anaphylactic sensitivity to guinea pigs; it has been demonstrated that the sensitivity induced in rabbits to foreign proteins while incorporated in Freund's adjuvant is of the usual anaphylactic or foreign protein type. The animals simultaneously develop a tuberculin sensitivity due to the *M. tuberculosis* organisms injected but this in no way alters the quality of the sensitivity developed to the foreign proteins. The persistence of the skin reactions to the higher concentrations of the serum antigens appears to be a reaction to the marked necrosis of tissue that occurs in these highly sensitized animals to the concentrated antigens rather than to any fundamental qualitative difference in the type of reactivity induced in the animals.

Received June 25, 1951. P.S.E.B.M., 1951, v77.

Effects of Oral and Intrasplenic Administration of Cortisone.* (18898)

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It has been found in our laboratories that

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* The cortisone acetate suspension was generously supplied by the Merck Corp. through the courtesy of Dr. A. Gibson. We are indebted to Dr. J. J. Schneider, Division of Endocrine and Cancer Research, Jefferson Medical College, for preparing the cortisone hemisuccinate used in this work. The technical assistance of Mrs. M. Sammaritano is gratefully acknowledged.

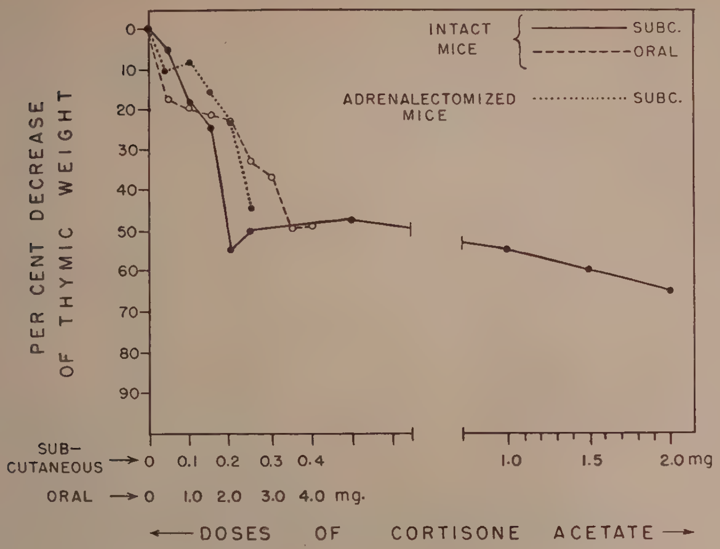

FIG. 1. Effect of a single dose of cortisone acetate on thymic weight of the mouse.

liver(1,2) and other tissues(1) inactivate cortisone *in vitro*. On the other hand, good therapeutic effects have been obtained with oral administration of cortisone in the treatment of rheumatoid arthritis(3-6). It was therefore deemed of interest to compare the effects of subcutaneous, intrasplenic and oral administration of cortisone in the mouse, in order to evaluate the role of the liver in inactivating this compound *in vivo*.

Methods. The effect of commercial cortisone acetate and of cortisone hemisuccinate upon the thymic weight of intact and adrenalectomized male mice (5 to 6 weeks old) was studied. The cortisone hemisuccinate was dissolved in a saline phosphate buffer solution at a pH of 7.4, 0.1 ml of the solution containing 3.2 mg of cortisone hemisuccinate (2.5 mg free cortisone). A comparison was made of (a) intrasplenic and subcutaneous routes and (b) oral (stomach tube) and subcutaneous routes. The weight loss of the thymus offered an accurate quantitative measurable endpoint.

For intrasplenic injection, the mice were anesthetized with ether, and the spleen was exposed by laparotomy. Oral administration was by stomach tube using either a piece of No. 4 ureteral catheter, or polyethylene tubing (.034 inch inside diameter). Because in the intact animal the operation and the passing of the stomach tube respectively could cause an alarm reaction, and a decrease of thymic weight through endogenous cortical hormones, all control animals were subjected to the same procedures receiving 0.85% NaCl intrasplenically, subcutaneously or by stomach tube respectively. The animals were autopsied at the end of 14 hours or at the termination of the experiment, and the wet weight of the thymus

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TABLE I. Effects of Repeated Daily Administration of Oral and Subcutaneous Cortisone Acetate on Thymic Weight of Mice.

Procedure	No. of animals	Body wt (g)	Thymic wt (mg)	Thymic wt (mg/100 g)	% change (thymic wt)	"P"*
A. Control I (0.85% NaCl 0.1 ml S.C. daily × 5 days)	10	18.8	50.9	272.9 ± 20.90	—	—
B. Cortisone acetate (0.25 mg S.C. daily × 5 days)	10	17	6.9	41.1 ± 3.55	-84.9	<.001 (B-A)
C. Control II (0.85% NaCl 0.1 ml daily × 5 days orally)	10	19.3	48.1	247.4 ± 18.14	-9.5	<.4 (C-A)
D. Cortisone acetate (0.25 mg orally daily × 5 days)	10	17.8	20.8	116.4 ± 9.53	-53	<.001 (D-C)

* Students Method, Fisher, R. A., Statistical Methods for Research Work, 4th ed., Edinburgh 1932.

was determined on a Roller-Smith torsion balance. The comparative effects of a single dose and of repeated daily doses when given by the oral and subcutaneous routes were studied.

Results. A. *Oral and subcutaneous administration of cortisone acetate.* Fig. 1 is a dose-response curve of single subcutaneous and oral doses of cortisone acetate upon the thymic weight of male mice at the end of 14 hours. Ten animals were used with each dose. The minimal single subcutaneous dose of cortisone acetate which caused a statistically significant drop in thymic weight was found to be 0.2 mg as compared to 3.0 mg when the compound was administered by the oral route. Thus, under the conditions of our experiment, a single dose of cortisone acetate was found to be about 10 to 15 times as effective when administered by the subcutaneous as compared with the oral route.

The sensitivity of adrenalectomized mice to a single subcutaneous dose of cortisone acetate did not differ from that of intact mice.

The effects of administration of daily oral and subcutaneous doses of cortisone acetate for a period of 5 days are illustrated in Table I. Whereas cortisone acetate was effective when administered by either route, the thymic involution was more marked in those animals receiving cortisone by subcutaneous injection. It is interesting to note that the total oral cortisone (1.25 mg) administered in this experiment was not effective when given as a single dose.

B. *Oral and intrasplenic administration of cortisone acetate and cortisone hemisuccinate.* (Table II). A single intrasplenic injection of 2.5 mg of cortisone acetate (= 2.21 mg cortisone) was as effective as the same dose given subcutaneously. Histological examination of the livers of the animals receiving this large dose of cortisone acetate intrasplenically revealed extensive focal necrosis. Therefore the effect of a buffered saline solution of cortisone hemisuccinate containing 3.2 mg of cortisone hemisuccinate (= 2.5 mg cortisone) per 0.1 ml of solution was studied. There was no difference in the thymic response following the intrasplenic administration of this compound as compared with the subcutaneous route. When a smaller dose of cortisone acetate (0.25 mg) was used, no effect on the thymic weight was observed following the intrasplenic administration, but a significant drop followed the subcutaneous injection of the same dose. Histological studies of the livers of these animals failed to reveal any pathological changes.

Discussion. Early experimental work by Heilman and Kendall(7) indicated that cortisone extracted from adrenal cortical tissue was physiologically active when fed to small laboratory animals. These investigators found that lymphosarcoma tumors failed to develop and well-developed tumors regressed rapidly in mice receiving 0.3 mg of Compound E daily in their drinking water. Recently, Emerson (8) has reported the effects of oral and subcutaneous cortisone on the development of experimental lymphosarcoma, the volume of the tumor following the administration of a single dose by either route being used as an

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7. Heilman, F. R., and Kendall, E. C., *Endocrinology*, 1944, v34, 416.

8. Emerson, G. A., *Trans. N. Y. Acad. Sciences*, 1951, v13, 92.

TABLE II. Effects of Subcutaneous, Intrasplenic and Oral Administration of Cortisone (Single Dose) on Thymic Wt of Mice (14 Hr).

					Relative		
Exp No.	Procedure	No. of animals	Body wt (g)	Thymic wt (mg)	Thymic wt (mg/100 g body wt)	% change of relative thymic wt	"P"*
1. A.	0.85% NaCl 0.1 ml subcutaneously (control 1)	10	18.6	73.2	393 ± 20.37		
B.	Cortisone Ac. (2.5 mg)† subcutaneously	10	19.6	43.8	222.1 ± 18.59	-43.4	<.05 (A-B)
C.	0.85% NaCl 0.1 ml intrasplenically (control 2)	10	16.4	55.7	337.5 ± 33.41	-9	<.2 (A-C)
D.	Cortisone Ac. (2.5 mg)† intrasplenically	10	17.3	35.8	210.4 ± 29.05	-37.6	<.01 (C-D)
2. A.	0.85% NaCl 0.1 ml subcutaneously	10	19.7	55.6	280.6 ± 19.67		
B.	0.85% NaCl 0.1 ml intrasplenically (control)	10	18.5	51.2	277.3 ± 14.49		>.9 (A-B)
C.	Cortisone hemisucc. (3.2 mg)‡ subcutaneously + 0.85% NaCl 0.1 ml intrasplenically	10	18.3	38.9	211.8 ± 25.05	-23.6	<.05 (B-C)
D.	Cortisone hemisucc. (3.2 mg)‡ intrasplenically 0.85 % NaCl 0.1 ml subcutaneously	10	19	38.7	202.7 ± 23.21	-26.9	<.02 (B-D)
3. A.	0.85% NaCl 0.1 ml subcutaneously	10	19.5	60.9	313.1 ± 22.50		
B.	Cortisone Ac. (0.25 mg) subcutaneously	9	20.6	38	183.3 ± 9.96	-41.4	<.001 (A-B)
C.	Cortisone Ac. (0.25 mg) orally	10	18.2	51.6	287.2 ± 30.09	-8.2	<.2 (A-C)
D.	Cortisone Ac. (0.25 mg) intrasplenically	10	19.7	66.5	339.1 ± 30.53	+8.5	<.5 (A-D)

* Student's Method, Fisher, R. A., Statistical Methods for Research Work, 4th ed., Edinburgh 1932.

† Equivalent to 2.21 mg free cortisone.

‡ Cortisone hemisuccinate solution 0.125 cc = 3.2 mg of cortisone hemisuccinate = 2.5 mg of free cortisone (phosphate buffer pH = 7.4).

index of cortisone activity. She found cortisone to be 5 to 10 times more active by the subcutaneous than by the oral route. Our results are in agreement with her findings, in as much as we found the minimal effective oral dose to be about 10 to 15 times that of the parenteral.

In our experiments small doses of cortisone, which exert a significant thymolytic effect when given by subcutaneous route, are ineffective either by intrasplenic or oral route. This suggests that the inactivation of cortisone by liver tissue previously observed *in vitro*(1,2) also occurs *in vivo*. With larger doses of cortisone, limitations of the capacity of the liver to inactivate the compound may permit enough to pass into the systemic circulation to produce a maximal effect upon the thymus.

The possible role of extrahepatic tissues in the inactivation of cortisone *in vivo* cannot be evaluated at present. The occurrence of this phenomenon in *in vitro* experiments has been demonstrated(1).

Clinical observations with orally administered cortisone have been reported by Freyberg(3), Hench(4), Boland and Headley(5), and Engleman *et al.*(6). In some cases the oral dose necessary to obtain a clinical response appeared to equal the intramuscular dose, whereas in others oral doses had to be up to 20 to 30% larger than intramuscular doses. Our studies on the effects of small daily doses of cortisone upon thymic weight duplicate clinical methods of administration. The animals receiving the drug subcutaneously showed a higher degree of thymic involution but oral administration was also quite effective. The fact that the total amount of orally administered cortisone was ineffective when given as a single dose suggests that a cumulative effect may be involved in the physiological action of cortisone.

Summary. (1) Thymic weight loss in 14 hours offers an accurate quantitative endpoint by means of which to measure the action of cortisone when administered by different routes. (2) Under conditions of our experiment, cortisone was much more effective when administered by subcutaneous route as compared with the oral or intrasplenic route. The subcutaneous:oral ratio is about 1:15. (3) Sensitivity of adrenalectomized mice to the thymolytic action of cortisone did not differ from that of intact animals. (4) Administration of small doses of cortisone, which caused thymic involution when given by subcutaneous route, failed to do so when given by intrasplenic or oral route. This suggests that inactivation by the liver, previously found in *in vitro* experiments, also plays a role *in vivo*. If the dose administered through the portal routes is large, sufficient amounts escape inactivation to produce a maximal effect upon the thymus.

Received June 25, 1951. P.S.E.B.M., 1951, v77.

Effect of ACTH and Cortisone on Phagocytosis. (18899)

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The experiments to be reported represent an attempt to determine the effect of adrenocorticotrophic hormone (ACTH) or cortisone treatment on the activity of the polymorphonuclear neutrophilic leukocyte in man. This study was prompted by the occasional progression of infection, possibly due to impaired phagocytic function, in patients undergoing treatment with these agents. For example, bacteremia with extensive abscess formation, without the usual systemic and local reactions, has been observed here and elsewhere. Some of these occurrences are cited by Mote(1). The general plan of the investigation was to use whole defibrinated blood from patients before and after treatment as a source of leukocytes, combined with virulent type I pneumococci and anti-type I pneumococcus rabbit serum. The organisms and antiserum were presumably unvarying, and any differences in phagocytic activity could be attributed to an effect, either direct or indirect, of the drugs on the leukocytes.

Methods and materials. The patients under examination were suffering from a variety of conditions which are indicated in Table I. A blood sample was obtained, defibrinated with glass beads, and tested before treatment was begun, and again after 4 to 21 days of therapy. Blood was taken at the same time each day; the patient was still under treatment when the last specimen was taken. The type I pneumococcus was maintained in cooked meat medium in the refrigerator for the duration of the experiment and no mouse or other passage of the organism took place. Ten to 12 hour cultures were made from this stock in 2% Bacto-tryptose, 0.5% glucose, 0.5% NaCl, 10% horse serum broth. The organisms were then neutralized to about pH 7.6, sedimented and suspended in 0.85% saline. The turbidity of the suspension in a 13 mm inside diameter cuvette was adjusted to 35% transmission at a wave-length of 620 Å in a Coleman Junior spectrophotometer. The antiserum stored in the frozen state was all of the same lot, small portions being thawed and diluted in 0.85% saline as needed.

Screw-cap vials, 6 x 1 cm, were loaded with 0.5 ml of blood, 0.1 ml of organism suspension and 0.1 ml of varying 2-fold dilutions of anti-

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1. Proc. 1st Clin. ACTH Conf., edited by John R. Mote, Blakiston Co., 1950.

TABLE I. Effect of ACTH or Cortisone on Phagocytosis.

Dilution of antiserum	1/80				1/160				1/320				Saline control			
	Pre‡		Post§		Pre		Post		Pre		Post		Pre		Post	
Patient	P.I.*	%†	P.I.	%	P.I.	%	P.I.	%	P.I.	%	P.I.	%	P.I.	%	P.I.	%
1. Rheumatoid arthritis cortisone 50 mg t.i.d. × 9 days	—‖		—		4.1	82	1.7	58	.98	52	.9	16	0	0	.06	6
2. Diffuse collagenic disease ACTH 15 mg q 6 hr × 7 days. 25 mg t.i.d. × 3 days	9.12	100	7.9	100	5.72	98	1.08	50	1.6	54	.48	26	.28	14	.08	8
3. Rheumatoid arthritis cortisone 50 mg t.i.d. × 7 days	—		9.10	92	2.56	72	1.08	32	.28	20	.40	22	.14	8	.06	6
4. Rheumatic fever cortisone 300 mg stat 100 mg daily × 9 days	7.04	94	4.9	70	1.62	50	1.08	36	.18	10	.08	8	0	0	.02	1
5. Bronchial asthma cortisone 100 mg daily × 10 days ACTH 100 mg daily × 7 days	7.3	92	—		1.3	47	7.2	100	.22	12	5.2	100	.06	6	4.6	100
6. Psychoneurosis cortisone 100 mg daily × 5 days	13.4	100	9.28	99	4.2	68	1.88	56	.32	18	.48	18	.18	12	.04	1
7. Rheumatoid arthritis ACTH 25 mg q.i.d. × 7 days	6.56	98	8.6	88	1.76	64	.70	30	.68	28	.20	8	0	0	0	0
8. Psychoneurosis ACTH 25 mg t.i.d. × 5 days	23	98	4.9	82	.67	55	.9	36	.56	32	.06	6	.14	6	.02	2
9. Infectious hepatitis cortisone 50 mg b.i.d. × 5 days	2.8	60	.58	24	.72	26	.48	20	.20	12	.24	10	.04	4	.04	4
10. Rheumatoid arthritis ACTH 25 mg t.i.d. × 7 days	8.54	96	.18	16					.16	12	0	0	.04	4	0	0

* P.I. = Avg No. of organisms per cell (phagocytic index). † % = % of neutrophiles taking up organisms. ‡ Pre = Blood taken before treatment. § Post = Blood taken after treatment. ‖ — = Too numerous to count.

serum starting at a dilution of 1:10. A saline control, without antiserum, was included. A 3 mm stainless steel ball was placed in the tube which was closed with a double layer of parafilm as a gasket under the screw cap. The tubes were rotated at 2 RPM end-over-end at 37°C for 20 minutes. Wright's stained films were prepared and the number of organisms taken up by 50 neutrophiles, as well as the number of participating neutrophiles was determined.

Results. It is apparent (Table I) that leukocytic activity is lower in the blood sample taken after treatment with ACTH or cortisone than in that obtained before treatment. In some cases this decrease is marked and in others it appears to be relatively slight. In only one instance (patient 5) did the activity increase; this appeared in the saline control also, and was shown to be due to the development of specific antibody during treatment, since the patient's serum undiluted or diluted 1:4, added to normal non-immune whole blood stimulated marked phagocytosis; no clinical manifestation of infection was noted. In another instance not shown in the table in which no pre-treatment blood was obtained, the patient developed multiple staphylococcal abscesses from which the causative organism was isolated. It was found that abundant phagocytosis of this organism took place in the patient's own blood. Since no comparable pre-treatment figures are available, it is obviously impossible to make any statement concerning the development of antibody, or the relative decrease in the activity of the phagocyte. Incidentally, this individual was receiving penicillin when the infection appeared, the organism was penicillin-susceptible, and regression of the abscesses occurred promptly when ACTH therapy was withdrawn.

Whether the consistently observed depression of phagocytic activity is of sufficient magnitude to account for increased susceptibility to infection is not certain although it might be at least a contributory factor. Alteration in complement content could explain this effect, but it has been shown(2) that, if anything,

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2. Pitner, G., Smith, L. C., and Calwell, C. A., *Bact. Proc.*, 1951, p88.

complement levels rise during ACTH treatment.

Summary. In 9 of 10 patients under treatment with ACTH or cortisone, the phagocytic activity of the neutrophilic leukocyte decreased. In the remaining case an increase was observed which was shown to be due to the development of specific antibody during treatment.

Received June 26, 1951. P.S.E.B.M., 1951, v77.

A Study of the Transfer of Serum Proteins into Tissue Injured by Tourniquet.* (18900)

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During the past 20 years, a large quantity of evidence has accumulated indicating that in traumatic shock there is a displacement of fluid from the blood into the tissue of the injured area. Blalock(1) reported that the loss of circulating volume could be accounted for by the gain in weight of the traumatized area. This observation has been supported by the work of Nickerson(2), Ashworth, Jester, and Lloyd(3), Swingle *et al.*(4), and others, and it has been concluded by most investigators in this field that the loss of plasma is the principal cause of the condition known as shock. Inasmuch as plasma is a complex substance consisting of several protein components as well as electrolytes and water, it is necessary to determine specifically the transfer of each of these substances in order to understand the role they play in the syndrome of shock.

This paper presents analytical data on the nature of the proteins found in injured and uninjured tissue and compares them with those found in serum.

General procedure. Partial ischemia in one hind leg of each of 6 anesthetized dogs was produced by binding with a tight tourniquet. The animals were anesthetized by intravenous injection of nembutal (0.6 cc of a 6% solution per kg body weight). The tourniquets were made tight by first wrapping about 10 feet of elastic bandage high up on the thigh, then a double loop of insulated wire which was drawn tight by twisting with pliers. This procedure produces neither complete nor reproducible degrees of ischemia, the variation being partially dependent on the size of the animal used. The bandage was left for 4 to 5 hours (Table I) and during the entire experiment small quantities of nembutal were administered when needed to keep the animal in deep anesthesia. Sometime during this period a relatively large quantity of the blue dye, T-1824, was injected via jugular vein. This was done for blood volume determinations and also to see whether dye-tagged albumin could be traced into the tissues. Rawson(5) has shown that 8 to 14 molecules of dye are bound by one molecule of albumin. In these experiments the number of moles of dye used was much less than the number of moles of albumin in the blood so that the albumin should be far from saturated and the binding relatively complete.

At about 1½ hours after release of the tourniquets the animals were exsanguinated and in one case (Dog No. 2) both femoral

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* This investigation was supported, in part, by the U. S. Navy, Office of Naval Research.

1. Blalock, A., *Arch. Surg.*, 1930, v20, 959.

2. Nickerson, J. L., *Am. J. Physiol.*, 1945, v144, 429.

3. Ashworth, C. T., Jester, A. W., and Lloyd, G. E., *Am. J. Physiol.*, 1944, v141, 571.

4. Swingle, W. W., Remington, J. W., Kleinberg, W., Drill, V. A., and Eversole, W. J., *Am. J. Physiol*, 1942, v138, 156.

5. Rawson, R. A., *Am. J. Physiol.*, 1943, v138, 708.

TABLE I. General Data on Leg Injury and Dye Injection.

			Time after application of tourniquet		
Dog No.	Wt, kg	Dye inj, g	Dye inj, min	Tourniquet removed, min	Exsanguinated, min
1	10.7	.0500	175	187	260
2	13.1	.0550	235	245	330
3	8	.0212	238	268	355
4	6.2	.0212	257	299	360
5	14.5	.0479	37	260	355
6	12	.0479	230	255	360

arteries were cannulated and perfused with saline in an attempt to wash out the residual blood. About 500 g of flesh (not skin) was excised from each. Proteins were extracted from each by first adding a buffer 0.02 M with respect to sodium phosphate and 0.15 M with respect to NaCl and chopping and grinding in a mortar or by subjecting to the action of a Waring Blendor, then clarifying by centrifuging at 3000 r.p.m. The proteins extracted by the two procedures did not differ significantly except that the blendor extracted more protein from a given quantity of flesh and the ratio of the quantity of albumin to other components was slightly lower.

Results. T-1824 dye in tissue. Gross examination of the flesh of the injured and uninjured legs revealed, by its strong blue color, the presence of dye in the injured but not in the uninjured tissue. In every case there was this marked difference in the appearance of the 2 legs, and the color was strongest in the flesh immediately under the tourniquet. However, the dye could not be extracted by homogenizing the tissue in buffered saline but was centrifuged down at 3000 r.p.m. with the tissue debris.

Serum proteins in tissue. Electrophoretic analysis of the proteins extracted in saline-phosphate buffer at pH 7.4 from the injured and uninjured tissue revealed a marked difference (Fig. 1 and Table II). In the extract from the uninjured tissue the component having the mobility of albumin amounted to from 2.8% to 7.1% of the total pattern area, whereas in the extract from the injured tissue from 8.2% to 27.8% was albumin (other evidence of the identity of this albumin is given in the following section). Similar differences in injured and uninjured tissue extracts were found in mice(6). In the experiment where the dog legs were perfused the amount of extracted albumin in the uninjured flesh was hardly perceptible (only 2 or 3% of the total pattern area) whereas in the injured flesh it was about 17%.

In all of these extracts from injured legs of dogs as well as those of mice(6) the absence of α- and β-globulins is very striking. It is impossible to determine the presence or absence of γ-globulin because of the proximity of its mobility to that of the normal tissue proteins but in the injuries dealt with here it appears that albumin is the only plasma protein that appreciably leaks through into the tissue after injury by tourniquet. The mobilities (Table II) of the albumins taken from the injured and uninjured legs appear, in some cases, to be significantly different, but the cause is not yet known.

Immunochemical identification of albumin. The methods of quantitative immunochemistry have provided additional criteria for the

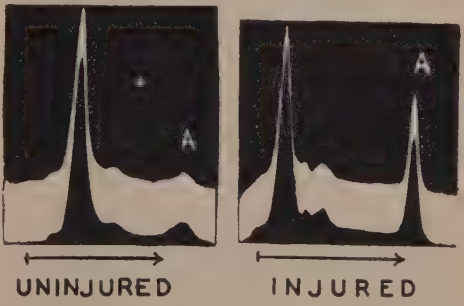

FIG. 1. Electrophoretic patterns of buffered saline extracts of tissues from contralateral uninjured leg and the leg injured by tourniquet of dog No. 4. (A = albumin).

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6. Moore, D. H., and Fox, Jr., C. L., *Nature*, London, 1950, v165, 872.

TABLE II. Electrophoretic Comparison of Buffered Saline (pH 7.4) Extracts from Injured and Contralateral Uninjured Leg Tissues.

	Uninjured leg			Injured leg			
Dog No.	Total pattern area, Arbitrary units	Albumin, % of total	Mob. alb., -10^{-5} cm^2 sec^{-1} volt^{-1}	Total pattern area, Arbitrary units	Albumin, % of total	Mob. alb., -10^{-5} cm^2 sec^{-1} volt^{-1}	Method of extraction
2	154	5.8	7.1	152	22	5.8	Mortar and pestle
2	1265	2.8	7.1	1830	16.8	5.8	Waring blendor
3	240	4.2	6.1	315	22.2	6.2	,, ,,
4	280	7.1	7	360	27.8	6	,, ,,
5	715	3.5	6.8	735	8.2	6.4	,, ,,
6	724	2.8	6.1	667	17.1	5.6	,, ,,

identification of proteins(7). Therefore the quantitative precipitin reaction was used to compare the albumin obtained from serum with that from injured leg extract.

An antiserum to dog serum albumin (DSA) which had been prepared some years previously in the laboratory of Dr. Michael Heidelberger was used. The antiserum was diluted 1 to 8 with 0.15 M NaCl containing 1:10,000 merthiolate as a preservative. For purposes of the precipitin reaction, electrophoretically-separated samples of dog serum albumin (DSA) and injured dog's leg albumin (DLA) were used. The DSA was diluted to 120 and 124 μg nitrogen per ml (2 dilutions of the same preparation were made) and the DLA was diluted to 136 μg nitrogen per ml with 0.15 M NaCl and stored at these concentrations, all subsequent dilutions being made from these stocks. Final dilutions were made in saline containing normal rabbit serum and were prepared immediately prior to addition to the antiserum. The reaction mixtures in duplicate, with appropriate blanks, were allowed to stand from 48 to 72 hours at 0-5°C in the icebox. Nitrogen was determined by the Markham modification of the micro-Kjeldahl method(8).

The results are summarized in Table III. From these data it must be concluded that the 2 albumins behave identically with respect to the antiserum to one of them, and therefore are chemically either the same or very similar.

TABLE III. Addition of Dog Serum Albumin (DSA) and Albumin from Injured Leg (DLA) to 1.0 ml (1-8) Antiserum to Dog Serum Albumin.

Preparation	Antigen N added, μg	Total N pptd, μg
DSA	4.96*	49*
DLA	5.44*	50*
DSA	8.3 †	61†
DLA	8.2	62
DSA	10.3	75
DLA	10.9	72
DSA	12	82
DSA	16 †	39†‡
DLA	16.3	37‡

* Double quantities were actually used.

† One and one-half times these quantities were used.

‡ Single determination.

Ultracentrifugal identification of albumin. Sedimentation constant determinations of albumin electrophoretically-separated from the serum and from tissue extract of the injured limb were carried out in an air-driven ultracentrifuge(9). The measured values which were made in the phosphate-saline buffer at temperatures between 20 and 30°C were corrected to the standard conditions of 20°C and a solvent of pure water.

The albumins from the serum and from the injured tissue were found to be indistinguishable, both having a sedimentation constant in the neighborhood of 4.5 S at concentrations of about 5 mg/ml.

Discussion and conclusions. The data presented here are believed to offer conclusive proof that a tight tourniquet applied to a hind leg of an anesthetized dog for a period of 4

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7. Kabat, E. A., and Mayer, M. M., *Experimental Immunochemistry*, Charles C. Thomas, Publishers, Springfield, Ill., 1948.

8. Markham, R., *Biochem. J.*, 1942, v36, 790.

9. Chiles, J. A., and Severinghaus, A. E., *Rev. Scient. Instruments*, 1940, v11, 71.

hours will, upon release, cause, as has been thought by many investigators, a transfer of serum albumin into the tissue affected by the tourniquet. A comparison of buffered saline extracts of tissue from injured and uninjured legs by means of electrophoresis revealed large quantities of plasma albumin in the injured tissue extracts in contrast to only traces in the contralateral uninjured tissue extracts. The albumin was further identified by immunochemical procedures and by its sedimentation constant in the ultracentrifuge. The fact that the albumin electrophoretically-separated from the tissue extracts reacted quantitatively with an anti-dog albumin rabbit serum in the identical manner as the albumin electrophoretically-separated from the dog serum would seem to indicate that the albumin in the injured tissue came from the serum.

The effect of the tourniquet seems to lower but not remove the barrier between the blood and the tissue because, of the plasma proteins, only albumin, which is the smallest molecule with the highest negative net charge, is transferred to any appreciable degree. The electrophoretic analyses also indicate that the loss of serum albumin is localized to the injured area. It is believed that the small amount of albumin (Table II) appearing in extracts from the uninjured legs comes from residual plasma in the capillaries. Perfusion of the legs with saline after exsanguination resulted in leaving in the control only a trace of material with the mobility of albumin. These data, therefore, support the idea that plasma albumin goes exclusively into the injured tissues.

Summary Electrophoretic, ultracentrifugal and immunochemical analyses of a protein extracted in buffered saline from tissues injured by the application of a tight tourniquet to one hind leg of the dogs indicate that large quantities of serum albumin enter the injured tissue. However, little or none of the α- and β-globulins enters the tissue. The dye, T-1824, injected intravenously before release of the tourniquet appears in the tissue of the injured leg only.

Received June 26, 1951. P.S.E.B.M., 1951. v77.

Biologic Assay of Adrenal Cortex Hormones by the Method of Unstained Cell Counts.* (18901)

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Schrek(1,2) found that compounds B and F and extracts of the adrenal cortex had a direct cytocidal action on rabbit lymphocytes *in vitro.* Feldman(3) observed a direct toxic action on lymphocytes by compound A and by adrenal cortex extracts. In the present study, the lymphocytotoxic action is used for the biologic assay of the adrenal cortex hormones.

Methods. The reagents tested were compounds B, E and F, an extract of the adrenal cortex and chromatographic fractions of the extract. All of these reagents were kindly provided by Drs. William J. Haines and Marvin H. Kuizenga of the Upjohn Company. Various quantities of the reagents, dissolved in methyl alcohol, were distributed to small, sterile test tubes 100 x 13 mm. The alcohol was evaporated *in vacuo* at room temperature. The tubes with the dried reagent were stored in the refrigerator. To prepare a cellular suspension, the thymus of a normal rabbit was removed aseptically. The tissue was chopped up with fine scissors in a medium of normal rabbit serum and Ringer's solution. The suspension was filtered through Monel metal wire screen.

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* Published with the approval of the Chief Medical Director, Veterans Administration. The statements and conclusions published by the author are the result of his own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

1. Schrek, R., *Endocrinology,* 1949, v45, 317.
2. Schrek, R., Proc. Soc. Exp. Biol. and Med., 1951, v76, 557.
3. Feldman, Joseph D., *Endocrinology,* 1950, v46, 552.

The cellular suspension was distributed to the test tubes with the dried reagent. The tubes were incubated in a water bath at 37°C and were shaken during incubation at the rate of 200 times per minute to prevent settling of the cells. The number of viable cells was determined in the treated and untreated suspensions. First, a solution of safranine 1:4000 in Tyrode's solution was added to the suspensions. A drop of the mixture was placed in a hemocytometer and the number of unstained cells was counted. The cells which resisted staining were considered to be viable.

The original suspension usually contained about 100,000 viable cells per cubic millimeter. After incubation for 24 hours the number (C) of viable cells in the control suspension without reagent was approximately 50 to 70% of the original number. When the percentage survival was less than 50%, the experiment was not considered satisfactory and the data were discarded. In the suspension treated with reagent, the number of viable cells may be represented by R. The per cent effect was calculated by the formula

$$100\ (1-\frac{R}{C}).$$

The results obtained were represented in a probit or dose-effect curve (Fig. 1) with the abscissa, the logarithm of the dose of the reagent in the individual test tubes and the ordinates, the per cent effect in probit units. The dose producing a 50% effect was interpolated from the graph and was termed the cytocidal unit.

Compound F, E, and B. The dose-effect curve for compound F is curvilinear (Fig. 1) and appears to approach a maximum of 80%. Even large doses (200 γ) failed to kill all the cells in the suspension within 24 hours. A dose as small as 0.01 γ produced an appreciable (34%) effect. The cytocidal unit or the dose that produced a 50% effect was, in this experiment, .02 γ.

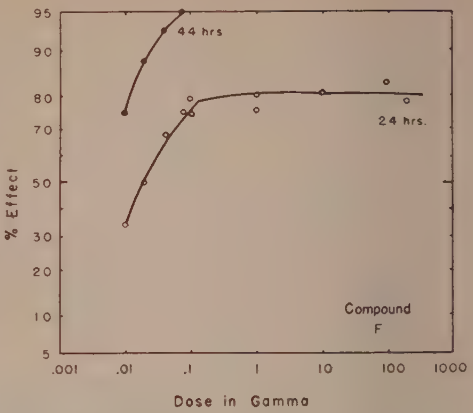

FIG. 1. Dose-effect curve showing % effect produced by 17-hydroxycorticosterone on rabbit lymphocytes incubated at 37°C for 24 and 44 hr. Abscissa shows log of the dose, and ordinates, the % effect in probit units. The % effect was calculated by the formula 100 (1–R/C) where No. of viable cells after incubation is represented by R for the treated and C for the control suspension.

The determinations of the cytocidal units of compound F in different experiments showed moderate variability (Table I). The arithmetic mean of the cytocidal units was 0.027 γ and the geometric mean, 0.024 γ. For convenience, 0.025 γ of compound F may be used as the standard cytocidal unit in the biologic assay of other hormones by the method of unstained cell counts.

In some experiments 44 hours of incubation was used as a period of incubation. The percentage of viable cells in the control suspen-

TABLE I. Frequency Distribution of Cytocidal Units of Compound F as Determined in Different Experiments.

Cytocidal unit (γ)	No. of cases
.010–.014	1
.015–.019	3
.020–.024	4
.025–.029	1
.030–.039	1
.040–.049	1
.050–.059	1
Total	12
Arithmetic mean	.027 γ
’’ standard deviation	.015 γ
Geometric mean	.024 γ
’’ standard deviation	1.65

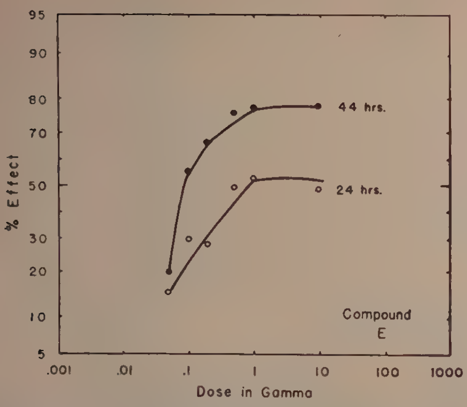

FIG. 2. The % effect produced by 17-hydroxy-11-dehydrocorticosterone on rabbit lymphocytes incubated at 37°C for 24 and 44 hr.

sion without reagent was 20 to 30% of that in the original unincubated suspension. The dose-effect curve in one experiment is shown in Fig. 1. At 44 hours, the per cent effect produced by any dosage of compound F was greater than at 24 hours of incubation. It appeared that the reagent had a slow and increasing effect on the cells. The 50% effect was produced by .016 γ.

The dose-effect curve for compound E incubated with cells for 24 hours (Fig. 2) is also curvilinear with a maximum effect of 50% in contrast to 80% for compound F. It is, therefore, not possible to determine for E cytocidal units based on the 50% effect in 24 hours.

The dose-effect curve after 44 hours of incubation showed a 78% effect with 10 γ of compound E, and a 50% effect with 0.1 γ. From these results, it is evident that compound F had both a greater and more rapid biologic effect than compound E.

The dose-effect curves for compound B incubated with cells for 24 and 44 hours were similar to those for compound E. The effect produced by 10 γ in 24 hours of incubation was 55% for compound B as compared to 50% for E. The 50% effect after 44 hours of incubation was .1 γ for both E and B. There was, then, no appreciable difference between the effects produced by compounds B and E.

Adrenal cortex extract. The dried adrenal cortex extract was supplied and titrated by Dr. Haines by means of the glycogen deposition method and was expressed in weight equivalents of compound F.

The cytocidal unit was found to be .08 γ —F equivalents as compared to .025 γ for compound F itself. Evidently the biologic assay of the adrenal cortex extract by the method of unstained cell count did not check exactly with that obtained by the glycogen

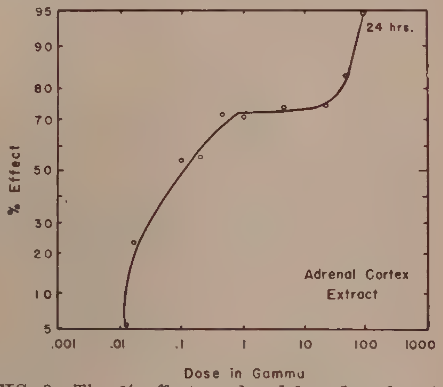

FIG. 3. The % effect produced by adrenal cortex extract on rabbit lymphocytes incubated at 37°C for 24 hr. Dose is expressed in wt equivalents of compound F as determined by the glycogen deposition method by Dr. Haines.

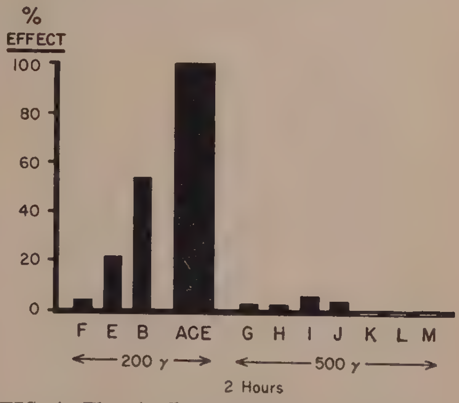

FIG. 4. The % effect produced by corticosteroids incubated with rabbit lymphocytes at 37°C for 2 hr. Preparations tested were 200 γ of the corticosteroids, F, E, and B, 200 γ of adrenal cortex extract (ACE) and 500 γ of the chromatographic fractions, G to M of the adrenal cortex extract.

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4. Feldman, Joseph D., *Yale J. Biol. and Med.*, 1950, v23, 114.

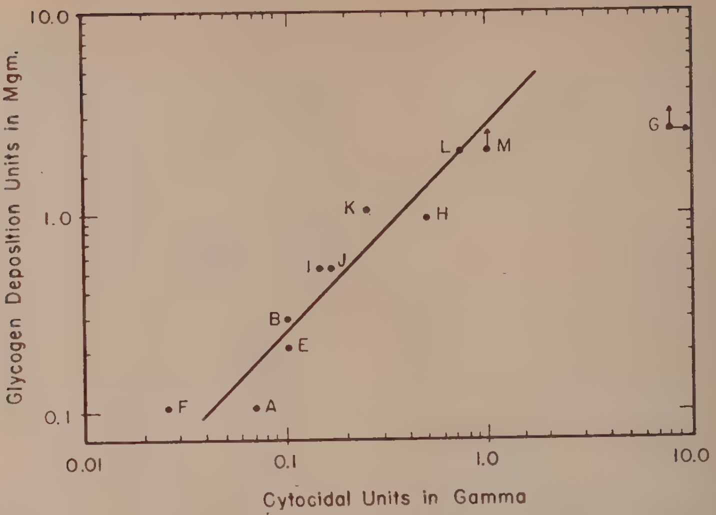

FIG. 5. Comparison of cytocidal units and glycogen deposition units for corticosteroids B, E and F, for adrenal cortex extract (A) and for chromatographic fractions G to M of the adrenal cortex extract. Cytocidal units were determined in this laboratory, and glycogen deposition units in the laboratory of the Upjohn Co.

deposition method. This is not surprising as the two methods test two different biologic properties of the extract.

The dose-effect curve for the extract (Fig. 3), is irregular in shape. With 10 γ —F equivalents of extract, the per cent effect was 75 which compared well with the 80% effect produced by 10 γ of compound F itself. With 100 γ —F equivalents of extract, there was a 95% effect although 100 γ of pure hormone F produced only an 80% effect.

In view of Feldman's report(3) that adrenal cortex extract had an immediate toxic effect on cells, and in view of the irregular dose-effect curve, a study was made of the toxicity of the extract after 2 hours of incubation at 37°C (Fig. 4). It was found that 200 γ —F equivalents of extract killed all the cells in 2 hours but 200 γ of compound F had no appreciable effect. Compound B did have a considerable effect (56%) and E a slight effect (22%). It may be concluded that not only the extract but also the pure hormones B and E in very large doses had rapid toxic effects on lymphocytes.

Comparison of two methods of biologic assay. Fig. 5 summarizes the findings obtained on the biologic assay of compounds B, E and F, the adrenal cortex extract (A in the figure) and of 7 chromatographic fractions of the extract (G to M). Dr. Haines prepared the chromatographic fractions and assayed the preparations by the glycogen deposition method. In this laboratory, the method of unstained cell counts was used for the biologic assay of the fractions.

According to both methods of assay, compound F was the most potent reagent tested with a cytocidal unit of .025 γ and a glycogen deposition unit of .1 mg. With the method of unstained cell counts and with 44 hours instead of 24 hours of incubation, compounds B and E had cytocidal units of 0.1 γ. With the glycogen deposition method, compound E was slightly more active with a unit of .2 mg as compared to .28 mg for compound B.

It is seen in the figure that the circles can be approximately represented by a straight line. Evidently, then, there is a fairly good correlation between the cytocidal unit and the glycogen unit.

Discussion. The mechanism of the delayed cytotoxic action of adrenal cortex hormones on the lymphocyte is not known. In view of the fact that compounds A, B, E and F, and not desoxycorticosterone affected by lymphocytes, it would seem that the delayed lymphocytocidal action may be a function of the C-11-oxygenated hormones.

Feldman(3) demonstrated that large amounts of adrenal cortex extract had an immediate cytotoxic effect on lymphocytes. The present study confirms this finding. Feldman observed in addition that the extract, in large doses, killed not only lymphocytes but many other types of cells and that the immediate cytotoxic effect took place at room temperature as well as at physiologic temperatures. In this laboratory, small and moderate doses of adrenal cortex extract had no effect on cells other than lymphocytes and had no effect on lymphocytes at room temperature. In these respects the immediate and delayed cytocidal effect appeared distinct.

To explain the relationships between the immediate and the delayed cytotoxic effect of adrenal cortex extract, it may be assumed that a compound in the extract affects two distinct structures or processes of the cell. According to this hypothesis, small and moderate doses of the adrenal cortex extract alter a specific physiologic process in lymphocytes incubated at 37°C and hasten their death. A corollary of this hypothesis is that this physiologic process is absent or not essential to other cells at 37°C and to lymphocytes incubated at room temperature. In contrast, large doses apparently act as a universal protoplasmic poison and affect many types of cells at a wide temperature range.

A similar hypothesis may be used for the effect of x-rays on cells. Small doses of x-rays seem to have a specific action on mature lymphocytes and mitotic cells. Large doses affect all living cells. This "dual mechanism" hypothesis for the action of adrenal cortex and x-rays, seems useful as it explains a number of diverse findings.

Summary and conclusions. The proposed method of assay consists of incubating cellular suspensions derived from the rabbit thymus with adrenal cortex preparations at 37°C for 24 and 44 hours. The number of viable cells in the incubated and treated suspensions were determined by the method of unstained cell counts. The cytocidal unit was defined as that amount of reagent which killed 50% of the cells as compared to a control, incubated suspension. The cytocidal unit was found to be 0.025 γ for 17-hydroxycorticosterone and 0.1 γ for corticosterone and cortisone.

Small doses of the adrenal cortex preparations had a delayed cytocidal effect whereas large doses of adrenal cortex extract and corticosterone had an immediate effect.

Received June 26, 1951. P.S.E.B.M., 1951, v77.

Progressive Growth of C3H Mouse Lymphosarcoma in CF$_1$ Mice Treated with Cortisone Acetate. (18902)

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The relationships of growth and regression of strain specific transplantable mouse tumors when implanted in alien strains of mice has been established by Lewis(1). It was shown that spindle cell sarcomas arising in inbred strains of mice would grow progressively and kill when implanted in mice of the strain in which they originated. When transplanted in mice of alien genetic constitution however, the tumors would grow and then regress. The sojourn of such tumors for four days in alien strain mice conferred immunity against subsequent implantation of the same tumor.

Similar studies have recently been made by Kidd and Toolan(2) who implanted C3H mammary carcinoma and lymphosarcoma

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1. Lewis, M. R., *Bull. Johns Hopkins Hosp.*, 1940, v67, 325.

2. Kidd, J. G., and Toolan, H. W., *Fed. Proc.*, 1949, v8, 360.

TABLE I. Effect of Cortisone Acetate Treatment* on Progressive Growth of Lymphosarcoma 6C3H-ED in CF_1 Mice.

	No. of mice	Died of tumor	Tumor regressed	Died during treatment
Cortisone treated	30	17	6	7
Untreated controls	37	2	35	0

* Cortisone acetate aqueous suspension 40 mg/kg/day subcutaneously for 10 days.

6C3H-ED into A strain mice. The temporary growth of these tumors in A strain mice conferred immunity against subsequent implantations of the same tumors. By appropriate experiments(3,4) it was demonstrated that this immunity was associated with a necrobiotic function of lymphocytes against the implanted tumor cells.

Stoerk(5) has shown in rats and mice that regression of lymphosarcoma during riboflavin deficiency is followed by an acquired resistance against reimplantation with the same tumor. This immunity was gradually lost over a period of one year, and was rapidly diminished when marked loss of normal lymphoid tissue was produced by the administration of cortisone or desoxypyridoxine.

The implication of lymphocytes in tumor immunity processes and in the destruction of tumor cells transplanted into alien strains of mice, suggested that the resistance which alien strain mice possess against heterologous tumors might be obliterated if marked destruction of lymphocytes could be effected at the time of implantation of the tumor. To test this possibility, experiments were made to determine whether intensive treatment with cortisone would render CF_1 mice susceptible to the progressive growth of lymphosarcoma 6C3H-ED. The results of preliminary experiments are presented.

Materials and methods. Young adult CF_1 (Carworth Farms) male mice weighing between 17.5 and 18.5 g were implanted subcutaneously in the right axillary region under light ether anesthesia with small fragments (10 to 12 mg) of the Gardner lymphosarcoma 6C3H-ED, using an 18 gauge trocar. All transplants were from tumor carried by serial passage in JAX C3H mice.

Cortisone acetate was given by daily subcutaneous injection at a dose of 40 mg/kg in 0.25 ml of distilled water. The first dose was given about 4 hours before implantation and was repeated once every day for a total of 10 doses. Implanted mice were palpated on alternate days to determine the growth behavior of the tumor.

All mice were fed Wayne Fox food *ad lib.* and had water available to them throughout the experiments.

Results. The implanted lymphosarcoma grew in all untreated CF_1 mice and became palpable on the fifth or sixth day after implantation, reaching a maximum size of 6000 to 7000 mm^3 in about 2 weeks; after which rapid regression took place. In contrast, the majority of cortisone treated mice grew progressive tumors which cause death of the animals between the twenty-first and twenty-fifth day following implantation. These mice at the time of death carried enormous tumors which had extended to the opposite flank, into the thoracic and neck region, and around the fore and hind legs on the side of the original implantation. The results of several experiments are combined and summarized in Table I.

It is seen in Table I that only 2 of 37 of the untreated CF_1 mice implanted with lymphosarcoma 6C3H-ED died of the tumor, whereas 17 of the 23 mice surviving cortisone treatment died of progressive tumor growth. Thus, treatment with massive daily doses of cortisone acetate reduces the natural strain resistance of CF_1 mice to this tumor. A highly significant difference was found to exist between cortisone treated mice and untreated controls when tested for significance

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3. Kidd, J. G., and Toolan, H. W., *Fed. Proc.*, 1949, v8, 373.

4. Kidd, J. G., and Toolan, H. W., *Fed. Proc.*, 1950, v9, 385.

5. Stoerk, H. C., *Fed. Proc.*, 1951, v10, 372.

by the method outlined by Gray(6).

Seven of the 30 implanted mice treated with cortisone died before the tumors reached appreciable size; 4 died on the eighth, 2 on the ninth, and 1 on the eleventh day following the beginning of treatment. It is presumed that these mice died as a result of the large repeated doses of cortisone since 4 out of 18 normal unimplanted male CF_1 mice, similarly treated in experiments made to ascertain lymphoid tissue reducing effects, died between the seventh and tenth day of treatment. Normal mice receiving 40 mg/kg of cortisone acetate subcutaneously for 10 consecutive days show at autopsy profound atrophy of the thymus, reduction in the size of the spleen and considerable loss of weight.

Discussion and summary. The data show that the resistance of alien strain (CF_1) mice to the progressive growth of lymphosarcoma 6C3H-ED (originating in C3H mice) is greatly impaired by intensive treatment with cortisone acetate. Seventeen of the 23 CF_1 mice which survived treatment with cortisone died of progressive growth of lymphosarcoma implanted from C3H mice, whereas only 2 of 37 untreated mice developed progressive tumors.

It should be pointed out that the course of cortisone dosage used to bring about the effects described were excessive, actually constituting a lethal concentration for some of the CF_1 mice. The nature of the change in resistance shown by these experiments has not been established, but it might be inferred that it is associated with the well known depression of normal lymphoid tissue and immune responses which large doses of cortisone induce.

Addendum. After the above manuscript was submitted for publication, a paper by E. L. Howes (*Yale Jour. Biol. and Med.*, 1951, v23, 454) came to our attention. This paper described the progressive growth of adenocarcinoma EO771 or 775 when transplanted in Rockland Swiss mice treated with cortisone.

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6. Gray, P., *Arch. Biochem.*, 1947, v13, 461.

Received June 29, 1951. P.S.E.B.M., 1951, v77.

Effect of Roentgen Irradiation upon Protein Absorption in the Mouse.* (18903)

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In a study of the effect of X-irradiation upon fat absorption in the mouse Mead, Decker and Bennett(1) showed that contrary to most earlier reports, no impairment of absorption could be demonstrated and that the results of previous workers could often be explained by radiation-induced changes in motility of the gastrointestinal tract.

In a continuation of these experiments we have now investigated the effect of X-irradiation upon protein absorption. The purpose of this study was to determine first, whether the previous findings represented an isolated case or could be generalized to cover other types of nutritive elements; and second, whether the damaged small intestine might permit the passage of appreciable quantities of material with molecular sizes larger than those of the amino acids. Under ordinary circumstances in normal animals, proteins are completely broken down before absorption. However, evidence has accumulated which indicates that in the irradiated animal intestinal permeability may be altered so as to allow the passage of bacteria(2). It was thought that the possibility of the absorption of intact protein could readily be demon-

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* This paper is based on work performed under Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

1. Mead, J. F., Decker, A. B., and Bennett, L. R., *J. Nutrition,* 1951, v42, 485.

2. Hammond, C. W., and Miller, C. P., *Ann. New York Acad. Science,* 1950, v53, 303.

strated by the use of iodinated proteins, since the iodinated amino acids released by hydrolysis are not rapidly reincorporated into serum proteins(3).

Materials and Methods. Human serum albumin iodinated with I^{131}† was used as the test protein in this study. To a solution of this protein was added an appropriate amount of bovine serum albumin (Cohn's fraction V)‡ to make a solution containing 35-37% total protein. The animals used for the study were female mice of the C.F. 1 strain, which had a mean weight of 27.5 g with extremes of 22.0 and 34.3 g.

During the first of the experiment, it was thought necessary to keep the mice on a protein-free diet§ for 12 hours before use. However, some of the animals lost weight during this time; and when it was found that absorption was in the same range for animals on a commercial stock diet, this initial procedure was abandoned and the animals used subsequently were given no preliminary change in diet.

For the determination of the rate of protein absorption, mice were fed a weighed amount (about 0.2 g containing 55-80 mg of protein) of the iodinated albumin solution measuring about 10,000 c/sec. on the counter in use. This material was delivered well into the esophagus using a 0.25-ml syringe with a bent No. 19 hypodermic needle ending in a blunt silver-solder tip. After the appropriate time had elapsed, the mice were sacrificed with chloroform. The gastrointestinal tract was dissected out, divided into 3 segments, and flushed with saline, as described previously(1), into 100 ml volumetric flasks. The suspensions were shaken thoroughly and allowed to stand for at least 15 minutes, after which time they were diluted to the mark with saline, shaken again, and allowed to settle for 5 minutes.

The activity in each segment was determined in triplicate by counts of basified (with NaOH) dried 0.5-ml aliquots of the washings. The amounts of protein recovered were then ascertained by a comparison of the activities in these samples with those in similarly treated samples of a standard prepared by diluting a weighed amount (0.05-0.1 g) of the test solution to 50 ml. The amount of protein absorbed could then be calculated from the difference between the dose given and the total amount recovered. Completeness of recovery was substantiated in 2 mice sacrificed 5 seconds after administration of the dose, the recovery values being 104% and 97%.

Serum protein-bound iodine was determined by comparing the activity of 0.1-ml aliquots of serum which were diluted with 0.5 ml saline, made alkaline with NaOH and dried, with that of precipitates obtained by the action of 5% trichloracetic acid on 0.1 ml aliquots of serum from the same animal. The

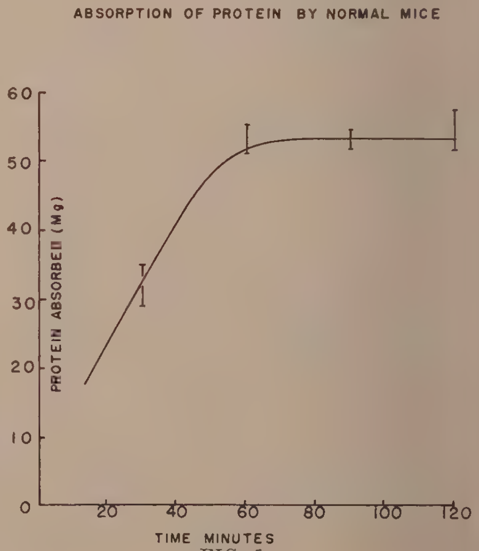

FIG. 1.

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3. Dixon, F. J., Bukantz, S. C., and Dammin, G. J., *Science,* 1951, v113, 274.

† Obtained as a solution from Abbott Laboratories. A micro-Kjeldahl determination made on one lot showed it to contain approximately 2% protein.

‡ Obtained from Armour Laboratories.

§ The diet consisted of 41% starch, 41% sucrose, 8% fish liver oil-corn oil mixture, 4% salt mixture (obtained from Nutritional Biochemicals Co.) and 6% celluflour (obtained from Chicago Dietetic Supply Co.). To condition the animals to the powdered diet, they were given the above diet with 20 g casein added per 100 g for two days previous to the protein-free diet.

TABLE I. Protein Absorption in Mice.

	No. of mice	mg protein in dose	% in stomach	Total protein absorbed in mg	% absorption of protein reaching small intestine
30-min test period					
Normal mice	7	55.8 ± 1.9	30.3 ± 5	31.9 ± 3.1	81.4 ± 2.2
2 hr post-irrad.	7	77.7 ± 2.4	53.5 ± 4.1	30 ± 2.6	84.1 ± 3.5
24 ” ”	8	79.3 ± 2.6	51.6 ± 5.8	32.4 ± 3.8	85.7 ± 3.3
96 ” ”	7	81.3 ± 1.6	41.8 ± 3.8	37 ± 3.6	77.6 ± 4.1
90-min test period					
Normal mice	10	79.1 ± 3.2	25.2 ± .9	53 ± 1.5	90.5 ± 1
2 hr post-irrad.	7	81.1 ± 1.4	39.8 ± 6.3	44.9 ± 5.1	91.4 ± 1.3
24 ” ”	8	78.4 ± 2.4	38.3 ± 5.2	44.7 ± 3.4	93 ± 1.5
96 ” ”	7	83.6 ± 2.9	28.3 ± 4.6	55.3 ± 4.6	92.2 ± .5

precipitates were centrifuged and washed 3 times with 5-10 ml portions of 5% TCA and were transferred to the planchets with 1.0 to 1.5 ml of saline and dried.

By means of the same technique, the standard was shown to contain about 98% protein-bound iodine. As a check on the adequacy of the precipitation and washing procedures, precipitations were carried out using normal mouse serum precipitated with the supernates remaining after the precipitation of sera from test animals which had received the iodinated protein. In each case, calculations were made of the percent of activity of the precipitate as compared with that of the whole serum.

Since a survey of the literature failed to reveal data on the rate of absorption of protein in the normal mouse, this investigation was undertaken as preliminary to that of the effects of X-irradiation.

Absorption was studied in normal mice at 30-minute intervals from zero to 120 minutes with a few points taken at 5 to 15 minutes. For the irradiated animals only the 30- and 90-minute periods were studied.

Irradiation of all mice was carried out at a distance of 50 cm and a dose rate of 41 r/min. for 600 r.[‖] This dose is somewhat greater than the mid-lethal dose in these laboratories.

Results. The absorption of protein by normal mice is shown graphically in Fig. 1, absorption of the test meal has essentially ceased after 60 minutes, and no further absorption could be observed to occur even up to 120 minutes, the unabsorbed portion of the dose being recovered from the stomach.

In all the irradiated animals for both test periods there was a marked retention of the dose in the stomach at 2 and 24 hours post irradiation (Table I, column 4). By 96 hours post irradiation, the emptying time of the stomach was approaching normality.

Despite this effect on the stomach emptying time, total protein absorption was only slightly reduced (Table I, column 5), and if absorption is calculated on the basis of the protein actually reaching the small intestine, no significant difference can be seen between the irradiated and control animals (Table I, column 6).

In Table II are recorded the results of the determination of protein-bound iodine in the sera of normal and irradiated mice and that found by precipitating normal serum with the supernates from precipitation of radioactive serum. It can readily be seen that there is no evidence for large-particle absorption either in the normal or in the irradiated animals since the activity of the precipitates in the 3 cases is of the same order of magnitude and apparently represents a constant error due to adsorption of small active molecules by the precipitate.

Discussion. The adequacy of I^{131} as a tracer for protein has recently been attested by Dixon and co-workers(3). However, it

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[‖] The radiation factors were: 250KV machine, operated at 15 Ma., with 0.21 mm CU inherent, 0.5 mm Cu parabolic, and 1.0 mm Al filters; HVL in the center of the field, 2.0 mm CU; 50 cm focal object distance.

TABLE II. Distribution of I^{131} in Blood Serum.

	No. of mice	Mean % of counts in TCA ppt	Range
Non-irradiated mice	28	1	.5–2.3
Irradiated mice	24	1	.2–3.8
Normal serum ppt with TCA supernatant from a radioactive mouse	12	1	.2–2.8

could be argued with some truth that in this case, since no whole protein was absorbed, the only rates measured were those of hydrolysis of protein and absorption of iodotyrosines. It is possible that the different amino acids are absorbed from the mixtures at different rates(4). Nevertheless it was thought that in this case, the iodotyrosines could be taken as typical amino acids in their absorption behavior and that the appearance of radio-activity in the blood would be a good measure of amino acid absorption in general. If significant differences in the absorption rates of normal and irradiated rats were found, they could be tken as symptomatic of the general permeability of the small intestine. Since no such differences have been found, it can be concluded that as seen in the case of fat absorption(1) no permeability differences are produced in this animal by this dosage of radiation and that in this case as well, motility and tonicity are the factors most affected. These factors have recently been investigated by Conard(5), who, using direct observation of the externalized intestine, found marked changes in motility following irradiation.

The study has also shown that no large quantities of whole protein are absorbed in either the normal or the irradiated animals, and that the ability of the irradiated animal to absorb protein in the normal fashion is essentially unimpaired for the periods and dosages studied.

Summary. The rate of protein absorption by normal mice has been measured using radioiodinated human serum albumin as a trace protein. No change in this rate could be shown after whole body X-irradiation of the mice although a change in tone and motility of the gastrointestinal tract was noted. No absorption of unhydrolyzed protein could be demonstrated.

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4. Orten, A. U., Koizumi, K., France, C. J., and Johnston, C. B., *Fed. Proc.*, 1951, v10, 390.

5. Conard, R. A., *Am. J. Physiol.*, 1951, v165, 375.

Received July 2, 1951. P.S.E.B.M., 1951, v77.

Effects of N-allylnormorphine on Cholinesterases. (18904)

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(Introduced by Edwin L. Smith.)

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Bernheim and Bernheim(1) found that morphine and apomorphine inhibit the cholinesterase of brain in sufficiently low molar concentration to enable them to speculate that this action of these drugs might be responsible for the cardiac and respiratory irregularities sometimes associated with their use. Wright and Sabine(2) confirmed the inhibitory effect of morphine on cholinesterase, and showed that Dilaudid and dihydrodesoxymorphine-D also inhibit the enzyme. They also reported that the effect of codeine varies considerably with the source of the enzyme (brain or serum). Eadie(3) reported that the inhibition of the cholinesterase of dog serum by morphine is a competitive one.

N-allylnormorphine (NAM) is a substance

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1. Bernheim, F. and Bernheim, M. L. C., *J. Pharmacol.*, 1936, v57, 427.

2. Wright, C. I. and Sabine, J. C., *J. Pharmacol.*, 1941, v72, 45.

3. Eadie, G. S., *J. Biol. Chem.*, 1941, v138, 597.

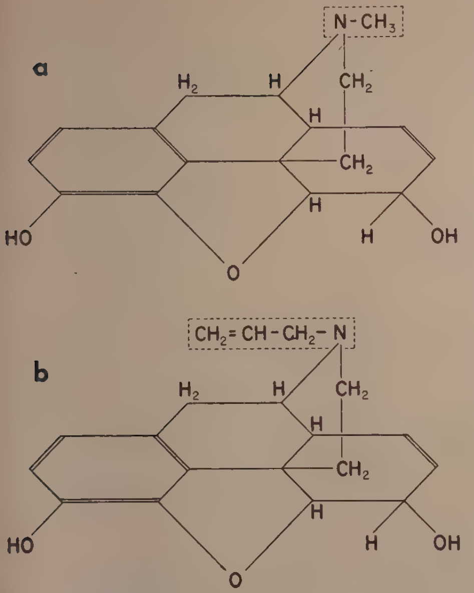

FIG. 1. a. Morphine. b. N-allylnormorphine. The dotted lines outline the different nitrogen substituents.

chemically very similar to morphine (Fig. 1), but which is antagonistic to the respiratory depressant, analgesic and stimulatory (cat) effects of morphine(4,5). Unna(4) found that although NAM abolished or prevented the analgesia due to morphine in mice, when administered alone it produced a shallow and transitory analgesia. Wikler(6) stated that in man large doses of NAM produce dysphoria, mild drowsiness, miosis, sweating and occasional nausea and circulatory disturbances.

Because of the chemical similarity and pharmacological antagonism of NAM and morphine, it was thought that NAM might be a useful tool in clarifying the relationship between the cholinesterase-inhibiting ability and the pharmacological effects of the morphine class of drugs.

Experimental. The sources of cholinesterase used were mouse brain homogenate, bovine red cell suspension and horse serum (Cutter Laboratories). The mouse brains were homogenized in Krebs-Ringer bicarbonate in a Potter-Elvehjem glass tissue homogenizer. The red cells were separated and washed three times in 0.9% NaCl, and suspended in Krebs-Ringer bicarbonate. In all cases the enzyme source was diluted with Krebs-Ringer bicarbonate so as to produce a CO_2-evolution in the uninhibited reaction of about 25 μl per 10-minute period. Variations from this value did not exceed 50%. All solutions were made up in Krebs-Ringer bicarbonate.

The enzyme and drug were placed in the main compartment of the Warburg vessel (Table I), and the acetylcholine was tipped in from the side arm at zero time. The flasks were gassed with 95% N_2— 5% CO_2. Readings were taken at 10-minute intervals for at least 60 minutes after tipping. Data were corrected for spontaneous hydrolysis of acetylcholine, possible metabolic CO_2 evolution, effects of the drugs on the enzymes and acetylcholine and thermobarometric changes.

The results of a single run are shown in Fig. 2. Only the straight-line portions of the curves are shown, since their slopes were used to calculate the percentage inhibitions presented in Table II.

Discussion. The rather wide variation in results makes interpretation difficult. This variation is believed to be a real variation in the susceptibility of the enzyme preparations to inhibition, since the agreement between the two flasks which contained the same drug in each run was always good.

There seems to be a general trend for NAM to be a slightly more potent inhibitor than morphine, but this is reversed in one case (0.0015 M on RBC).

Since NAM antagonizes most of the pharmacological actions of morphine, this seems to us to lend support to the idea that the ability of morphine to inhibit cholinesterase has little to do with its typical pharmacological effects, but that the side effects of morphine which Bernheim thought might be due to this ability, could indeed be so, since these effects are also produced by NAM.

It will be noted that the effects of these

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TABLE I. Set-up for a Single Warburg Run. Final drug concentration = 0.00075 M. Flasks gassed with 95% N_2–5% CO_2.

Warburg vessel No.	Side arm		Main compartment			
	AcCh, ml	KRb	Enzyme, ml	Morphine	NAM	KRb, ml
1	.25	—	.50	—	—	2.25
2	"	—	"	—	—	"
3	"	—	"	.75	—	1.50
4	"	—	"	"	—	"
5	"	—	"	—	.75	"
6	"	—	"	—	"	"
7	"	—	—	—	—	2.75
8	—	.25	—	—	—	"
9	.25	—	—	—	.75	2.00
10	—	.25	.50	—	"	1.50
11	.25	—	—	.75	—	2.00
12	—	.25	.50	"	—	1.50
13	—	"	"	—	—	2.25

drugs on the serum enzyme differ quantitatively from their effects on the enzymes from brain and red cells. This is not surprising in view of the fact that many other points of difference exist between these two types of enzyme(7,8).

Summary. The inhibitions of the cholinesterases of mouse brain, bovine erythrocytes

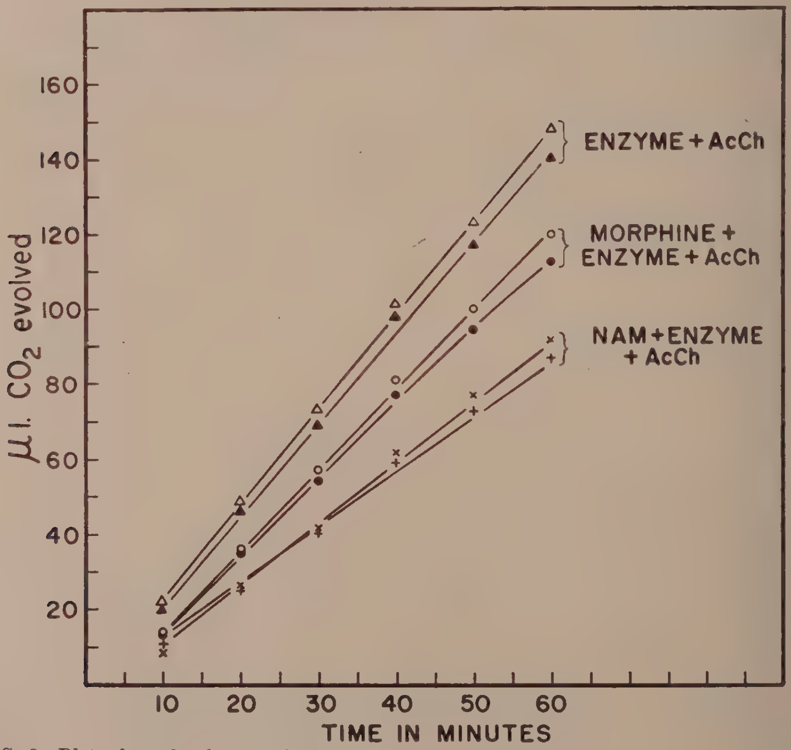

FIG. 2. Plot of results from a single run. AcCh = acetylcholine. Corrected for controls.

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TABLE II. % Inhibitions of Cholinesterases of Mouse Brain, Bovine Red Cells and Horse Serum by Various Concentrations of Morphine and NAM. Each value represents the average of the 2 values obtained in each run.

Drug concentration	Mouse brain		Bovine RBC		Horse serum	
	Morphine	NAM	Morphine	NAM	Morphine	NAM
.00038 M	8	18	17	23	48	58
	12	21	19	23	49	60
.00075 M	33	38	24	32	50	66
	26	40	23	28	49	84
.0015 M	43	60	60	54	64	79
	44	45	35	42	54	78

and horse serum produced by N-allylnormorphine were measured and compared to the inhibitions produced by morphine. N-allylnormorphine is at least as potent an inhibitor as morphine. The implications of this finding for the theory of morphine action are discussed.

We would like to express our thanks to Dr. Russell A. Huggins, Department of Pharmacology, Baylor University College of Medicine, and Dr. Edwin L. Smith, Physiology Department of this School, for their helpful discussions and criticisms of this work, and to the Merck Co. for generously supplying the N-allylnormorphine used.

Received July 3, 1951. P.S.E.B.M., 1951, v77.

The Electron Microscopy of Rabies Inclusion (Negri) Bodies. (18905)

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The great resolving power of the electron microscope together with our present ability to cut tissue sections thin enough for examination with this instrument has made it possible to visualize several viruses within their host cells. The present paper is a record of results of a preliminary attempt to recognize rabies virus in sections of diseased mouse brain. The Negri bodies that frequently are found in such tissue have long been studied under the optical microscope without, however, their developing any general agreement on the method of their development or their relation to the virus(1-7). First attempts to see Negri bodies with the electron microscope, using a modified smear technic, have given only very limited information about them(8).

For the present experiments, 3 to 4 weeks old Swiss mice were inoculated intracerebrally with strain 1449 of Street virus (obtained through the courtesy of Dr. Harry A. Rubin of the Communicable Disease Center Laboratory, Montgomery, Ala.) and sacrificed at 7, 9 and 12 days. After removal the brains were fixed in 10% formalin. Cross sections of the cerebral hemispheres at the level of the hippocampus were hardened for 3 days in 3%

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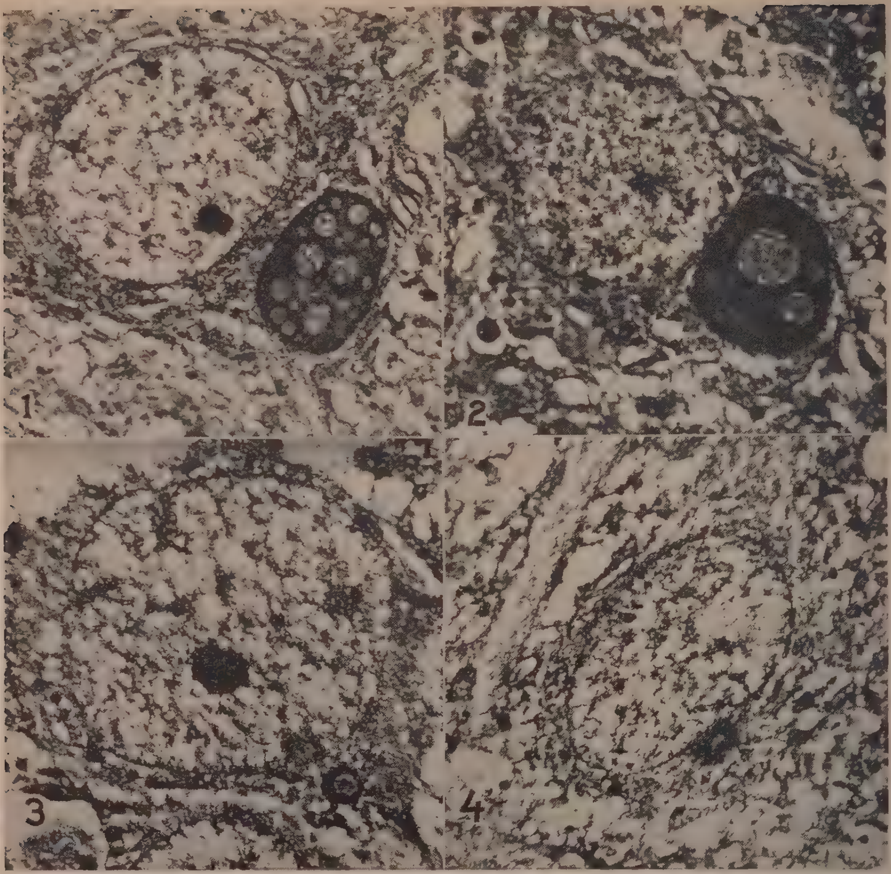

FIG. 1. Typical inclusion body containing vacuoles. Membrane of the nucleus, above to the left, is intact. In lower left corner of the figure are scattered granules suggestive of mitochondria by their size and density. Magnification = 3,800 ×.

FIG. 2. Inclusion body with 2 large vacuoles and characteristic, smaller, "daughter" vacuoles. Membrane of the nucleus in the upper part of the illustration has undergone dissolution. Magnification = 4,670 ×.

FIG. 3. Small inclusion with a single, central vacuole. Nuclear membrane is disrupted at several places. Magnification = 4,730 ×.

FIG. 4. Poorly demarcated, dense area with less dense central zone lying in the perinuclear cytoplasm. Nucleoli do not lie in the plane of section. Magnification = 5,000 ×.

aqueous solution of potassium bichromate, dehydrated in absolute alcohol, cleared in xylol and embedded in paraffin. Examination in the optical microscope of 5 μ thick sections cut from these blocks and stained with azure-eosin(9) accurately localized the large cell band of the hippocampus. Suitable tissue blocks selected in this way were immersed in xylol for 14 hours to remove the paraffin. From areas known to contain the large cells of the hippocampus small blocks, 1-2 mm in width, were then excised, embedded in methacrylate, thin-sectioned and prepared for the electron microscope by the methods outlined in previous papers. In addition 5 μ sections were cut from the methacrylate-embedded tissue, mounted on glass slides and the methacrylate removed by acetone. These thick sections were stained with azure-eosin and

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examined in the optical microscope. Control sections of the hippocampus from uninfected, 3-4 weeks old mice were prepared in the same way.

Comparisons with the optical microscope between tissue embedded in the original paraffin and those subsequently embedded in methacrylate do not reveal differences in the preservation of the cells. It should be mentioned, however, that using identical staining technics less eosin remains in sections of the methacrylate embedded tissue after dehydration and clearing.

In the hippocampus of moribund mice sacrificed 12 days after inoculation with rabies virus numerous large inclusion bodies can be identified in the optical microscope. In methacrylate embedded preparations examined by the electron microscope similar inclusions are readily recognizable. Fig. 1 shows a typical inclusion (Negri) body seen in the electron microscope. The body seems to contain small, irregular, poorly defined, dense granules and has multiple, oval vacuoles. Generally, as in this case, the vacuoles are filled by meshes of less dense material. Rabies virus as measured by filtration experiments (10,11) should be large enough to be clearly visible at this magnification. Distinct, dense, spherical granules are sometimes seen within vacuoles, but because they vary in size and are not consistently present it cannot be concluded that they are the elementary bodies of the virus. The larger forms probably are the "Innenkörper," the blue staining granules seen in the light microscope. The nuclear membrane of cells containing inclusions may be intact, as in Fig. 1, or partially or completely destroyed as shown in Fig. 2. The neurofibrillae in the cytoplasm of these cells, where they can be recognized, appear to lack an orderly reticular arrangement. This may be artifact, but sections through dendrites show a parallel arrangement; and this suggests that in the perinuclear region the neurofibrillae have variable courses, passing in and out of the plane of very thin sections.

In 7 and 9 days old lesions the electron microscope not only reveals the typical, multivacuolated inclusions just discussed but also more numerous, opaque objects too small to be recognized as inclusion bodies in the optical microscope. Fig. 3 shows one such body at the lower right, adjacent to the nucleus. The oval or spherical shape, density, presence of a central vacuole and perinuclear location suggest that these are small inclusions. Their number seems to preclude the possibility of their being the result of cutting through the periphery of large inclusions. Instead they may be early stages in development of the Negri bodies.

In 7 day old lesions similar, but poorly defined, dense bodies are found within the perinuclear cytoplasm (Fig. 4). Strands of cytoplasm can often be traced into them, suggesting a condensation or precipitation of opaque material in the cytoplasm. Such bodies are rarely encountered in 9 and 12 days old lesions. They are not present in control sections and perhaps are an especially early stage in the development of the inclusion body.

It has several times been suggested that the inclusions develop from nucleolar constituents extruded into the cytoplasm(1,5). The nucleoli of cells of both control animals and those containing inclusions vary greatly in size and shape and may lie against the nuclear membrane. Their position on the membrane is not, however, related to the position of the inclusion body, and there is no evidence from our electron micrographs that they contribute directly to the development of the inclusion body.

Summary. Electron micrographs of typical inclusion (Negri) bodies from the hippocampus of a mouse inoculated with rabies virus are shown together with smaller inclusions that may be early forms of the larger bodies. No particles have been recognized that could be identified with elementary bodies of this virus.

Received July 6, 1951. P.S.E.B.M., 1951, v77.

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Acetate-Replacing Factors for Lactic Acid Bacteria: Intracellular Distribution in Rat Liver. (18906)

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The existence in biological preparations of a group of chemically related substances that can replace acetate in its growth stimulatory effect for certain lactic acid bacteria has been demonstrated(1). Natural materials contain a microbiologically inactive form or forms that can be converted to 2 active substances by acid hydrolysis. Purification of one of these substances from liver has revealed that in catalytic amounts it is effective as an acetate-replacing factor(2). Concentrates of this factor are very active in the pyruvate oxidase factor assay of O'Kane and Gunsalus(3), suggesting that it is involved in oxidative decarboxylation of pyruvate.*

It has become aparent that certain types of enzymatic activity are associated with different parts of cells(4). Localization of acetate-replacing factor activity within liver cells would serve as a starting-point for a more detailed study of the function of these factors in animal tissues.

Experimental. Fractionation of cell components. Stock rats originally of the Wistar strain were fasted overnight, killed by decapitation, and the livers were quickly removed and chilled in ice-cold isotonic KCl. The livers were blotted, weighed, and homogenized in isotonic (0.25 M) sucrose in an all-glass apparatus(5). The homogenate was separated by the method of Schneider(6) into three fractions—nuclei, mitochondria, and a third fraction consisting of the submicroscopic particles and soluble components of the cells. The fractionation was carried out below 10°C.

TABLE I. Distribution of Acetate-Replacing Factor Activity in Fractions of Rat Liver Homogenates.

	Acetate-replacing factor activity	
Liver fraction	Total,* acetate units	Fraction of homogenate, %
Homogenate	7.5 (6 –8.9)	(100)
Nuclear fraction	1.3 (1 –1.6)	17.3
Mitochondrial fraction	4.8 (3.5–6)	64 (56–70)
Unfractionated residue	.9 (.7–1.2)	12
Unaccounted for	.5	6.7

* Per mg of fresh liver or its equivalent. One acetate unit is equivalent to the growth response of 1 mg of sodium acetate(1). Average values obtained with the livers of 4 rats are presented and the range of the experimental determinations is given in parentheses.

Assay of acetate-replacing factor activity. The medium and procedure employing a strain of *Streptococcus lactis* as the assay organism have been described(1). Samples of the various fractions and of the homogenate were autoclaved at 120° for 3 hours with an equal volume of 6 N sulfuric acid to release acetate-replacing factor activity(1). The acid hydrolysates were neutralized and assayed. The results are presented in Table I.

Discussion. In order to determine the distribution of acetate-replacing factor activity within liver cells, the latter were separated into 3 fractions by means of the cell fractionation technic. These fractions were the nuclei, mitochondria and a third fraction consisting of the submicroscopic particles and soluble components of the cell. Cytological examination indicated that the fractions consisted mainly of the components mentioned. Moreover, it was shown that the succinoxidase

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activity of the rat liver homogenates was associated mainly with the mitochondria, as had been previously demonstrated(7).

Having established that the liver cells had been separated into distinct particulate components, it was possible to determine the distribution of acetate-replacing factor activity in the cells. The results reported in Table I indicate that approximately 64% of the acetate-replacing factor activity of rat liver is associated with the mitochondria. This finding is in conformity with the apparent role of acetate-replacing factors in pyruvate oxidation and the fact that mitochondria are centers of respiratory activity in liver cells(7).

Summary. The major portion of the acetate-replacing factor activity of rat liver homogenates has been demonstrated to be associated with the mitochondria.

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Received July 9, 1951. P.S.E.B.M., 1951, v77.

Endocrine Influences on Proteinuria in the Rat: Effect of ACTH.* (18907)

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The kidney enzyme renin produces an intense though transient proteinuria when injected intraperitoneally into the normal rat (1). It has been shown that bilateral adrenalectomy abolishes this proteinuria(2), and that it is restored in the adrenalectomized rat by the injection of suitable doses of cortisone, desoxycorticosterone acetate and aqueous adrenal cortex extract(3). Hypophysectomy similarly abolishes renin proteinuria. Cortisone is effective in restoring the ability of the hypophysectomized rat to respond to renin with proteinuria while desoxycorticosterone acetate is not(4). The normal, spontaneous proteinuria of the male rat is reduced by bilateral adrenalectomy(3), and by hypophysectomy (4). Cortisone and desoxycorticosterone acetate restore this proteinuria to normal levels in the adrenalectomized rat but fail to do so in the hypophysectomized animal.

In order to define further the endocrine influences on certain types of proteinuria in the rat, the effect of various doses of pituitary adrenocorticotropic hormone (ACTH) on renin proteinuria and the spontaneous proteinuria of normal male rats has been studied.

Methods. Animals: Male albino rats of the Slonaker-Addis strain were used in these experiments.

Urine collections: All animals were taken from the colony at 4 P.M. and placed in individual urine collection cages overnight. Seventeen hours later the overnight urine collection period was terminated. The protein excreted during this overnight period is referred to as the animal's spontaneous or "base" proteinuria. During the overnight period the animals received only a 15% solution of glucose in 0.4% sodium chloride containing 0.5% of a solution of B vitamins (Betaplexin). Following the termination of the overnight urine

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* This work was supported by grants from the United States Public Health Service and the American Foundation for High Blood Pressure.

Pituitary adrenocorticotropic hormone was supplied by Drs. Edwin Hays and Irby Bunding of Armour and Co., Chicago, Ill.

† Howard R. Hughes Medical Research Fellow.

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TABLE I. Effect of ACTH on Base and Renin Urinary Volume and Protein Excretion.

	No. of observations	Mean body wt, g	Mean base urinary vol, ml/17 hr	Mean base protein excretion, mg/hr	Mean urinary volume after renin, ml/hr	Mean proteinuria after renin, mg/hr
Dose schedule A						
Control	29	189	45.4 ± 8.6‡	.85 ± .31	6.14 ± 1.74	44.4 ± 22.2*
ACTH-Renin, interval 1 hr	37	209	42.4 ± 7.9	.79 ± .18	5.97 ± 1.79	70.6 ± 27.2*
ACTH-Renin, interval 3 hr	26	”	”	”	5.60 ± 1.65	41.7
Control	10	300	62	1.5	4.9 ± 1.4	76
ACTH	10	300	60	1.1	6.4 ± 1.8	77.5
Dose schedule B						
Control	6	196	46	.82	7.7 ± .3†	63 ± 22.4*
ACTH	6	194	45	.89	5 ± 1.3†	100.1 ± 13.2*
Dose schedule C						
Control	30	221	52.1	.71 ± .28*	5.60 ± 1.67*	57 ± 28.9*
ACTH	37	227	45.5	.91 ± .15*	2.64 ± 1.21*	91.9 ± 32.9*
Dose schedule D						
Control	12	244			6.1	57.4
ACTH 3 mg	12	245			4.8	51.5
Control	12	220			6.5	62.2
ACTH .25 mg	12	220			6.7	56.1

* $P < 0.001$; † $P = 0.01–0.001$; ‡ Figures following the means are standard deviations.

collection period, the animals had their bladders emptied by allowing them to sniff ether lightly while pressure was exerted over the bladder area. All animals then received 4 Goldblatt dog units of hog renin[§] (purity 50 units per mg of nitrogen) in 4 ml of 0.85% sodium chloride solution intraperitoneally. The animals were then placed back in the metabolism cages and their urines collected during the next 60 minutes. Details of the methods for accurate rat urine collections are described elsewhere(1).

ACTH was administered subcutaneously in varying dosages and according to a variety of time schedules as described below. The dose of ACTH is expressed in terms of equivalent mg of the Armour Standard (LA-I-A). Urinary proteins were determined by a modification of Kingsley's biuret method(6).

Results. The data are expressed as means. In most cases, the standard deviation is given. P values are obtained from Fischer's(5) table of t for small numbers of observations. P values of 0.01 or less are taken to indicate a significant difference between the means.

A. *Dose schedule A.* ACTH was first administered subcutaneously in doses of 0.40 mg dissolved in 0.5 ml of 0.85% sodium chloride solution. Injections were given at 12 o'clock noon and 5 P.M. on the day before renin administration, and 10 A.M. on the day of renin administration. Control animals received 0.5 ml of 0.85% sodium chloride solution at similar times. Thirty-seven experimental animals and all of the control animals received renin at 11 A.M. Twenty-six experimental animals were injected with renin at 1 P.M. In each group the urines were carefully collected for the 60 minutes immediately following renin administration. The results are given in Table I.

ACTH when administered according to dosage schedule A, to rats weighing approximately 200 g, resulted in a 65% increase in the amount of protein excreted in the hour following renin administration over and above that shown by non-ACTH treated controls. There was no significant change from the control value in the volume or protein content of the 17-hour overnight urine or in the volume of urine excreted in the hour following renin administration.

[§] Renin was generously supplied by Drs. Harry Goldblatt, Erwin Haas, and Hildegard Lamfrom.

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If too long a time was allowed to elapse between the last dose of ACTH and the administration of renin, no increase in proteinuria was noted. Thus, the proteinuria in response to renin was greatly augmented when the ACTH-renin interval was one hour, but was entirely absent if this period was extended to 3 hours. When ACTH was administered according to dosage schedule A, to rats of approximately 150 days of age and weighing 300 g there was no significant increase in renin proteinuria (Table I).

It appeared that the dose of ACTH employed above was sufficient to augment the proteinuria resulting from renin administration in rats weighing 200 g, but was too small to be effective in the 300 g animals. Consequently, ACTH was given in larger doses to a fresh group of animals.

B. *Dose schedule B.* The dose of ACTH was increased to 1.6 mg given subcutaneously at 12 o'clock noon and 5 P.M. on the day before renin injection, and 10 A.M. on the day of renin administration (Table I).

When the dose of ACTH was increased to 1.6 mg per injection, the amount of protein excreted in response to renin was increased by 63%. Thus, in spite of a 4-fold increase in the amount of ACTH administered, the increase in renin proteinuria was nearly identical to that found with the 0.40 mg dose. In this instance, however, there was a significant fall in the volume of urine excreted in the hour following the renin administration. No significant change occurred in the volume or protein concentration of the urine excreted during the 17-hour overnight period.

C. *Dose schedule C.* In order to determine whether still larger doses of ACTH given at intervals throughout the overnight collection period might augment the base proteinuria, dosage schedule C was undertaken. 4.8 mg of ACTH was given at 5 P.M. and 12 midnight of the day preceding renin administration, and at 7 A.M. on the day renin was given. The results are given in Table I. ACTH administered in this manner resulted in a 61% increase in the quantity of protein excreted during the hour following renin administration. In addition, a sharp fall in the volume of urine excreted occurred during this period, and further, the quantity of protein excreted during the 17-hour overnight period was significantly increased by ACTH administration in the time-dosage schedule indicated. The apparent fall in the 17-hour overnight urine volume in the ACTH treated animals is not a statistically significant one.

D. *Dose schedule D.* This schedule was designed to determine whether or not ACTH administered the day preceding renin administration was necessary if an increase in renin proteinuria was to be obtained. All animals were placed in metabolism cages overnight, as described above. The following morning one-half of the experimental animals received 0.40 mg of ACTH and one hour later were injected intraperitoneally with 4 units of renin in 4 ml of 0.85% sodium chloride solution. The remaining half of the experimental animals received 4.8 mg of ACTH subcutaneously and 10 minutes later were injected with four units of renin in 4 ml of 0.85% sodium chloride solution intraperitoneally. Control animals received 0.5 ml of 0.85% sodium chloride solution subcutaneously instead of ACTH. In all instances, urines were collected for 60 minutes following renin administration (Table I).

It appears that a single dose of ACTH, whether large or small, when administered in the manner described above, will not produce an increase in protein excretion in the hour following renin injection. In rats of our colony single injections of ACTH in doses up to 25 mg have failed to produce a consistent eosinophile fall. It was not until 9.6 mg of ACTH was injected in 3 successive doses over a 17-hour period, that a satisfactory eosinopenia was produced. While the volume of urine excreted during this hour appears to be diminished in the animals given 4.8 mg of ACTH, this fall is not a statistically significant one.

Discussion. When given in the proper dose and at appropriate time intervals, ACTH is capable of markedly augmenting the intense transient proteinuria that occurs in the normal rat following renin administration. A less spectacular, nevertheless, significant increase in the normal, spontaneous proteinuria of rats occurs following suitable doses of ACTH. If the animal is too large, or the administration of ACTH is not suitably timed, one may fail to observe any significant change from the normal in protein excretion. Though it might seem that the sharp fall in urinary volume in the hour following schedule C, renin administration was a manifestation of posterior pituitary contamination of the ACTH used, this possibility was discarded following assay of our ACTH preparations for anti-diuretic substances.¶ Further, we have recently demonstrated that a similar fall in post-renin urinary volume occurs in cortisone-treated rats. Other preliminary studies in our laboratory indicate that the fall in urine volume in ACTH and cortisone-treated animals given renin may be a result of actual renal tubular damage with plugging of the tubular lumina by protein casts. Along similar lines, Masson, Corcoran, and Page have reported renal damage in rats treated with saline, desoxycorticosterone acetate, and renin(7).

The mechanism by which ACTH augments proteinuria in the rat is not indicated by the data presented here.

Summary. 1. Normal rats respond to the intraperitoneal injection of renin with an intense, transient proteinuria. ACTH administration results in a 60 to 65% increase in proteinuria in the hour following renin administration over and above that shown by non-ACTH treated controls. 2. The normal, spontaneous proteinuria of the male rat is significantly increased by ACTH administration.

¶ The assays were performed by Dr. Irby Bunding of Armour and Co., Chicago, Ill.

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Received July 9, 1951. P.S.E.B.M., 1951, v77.

Effect of Intranasally Administered Immune Serum on Influenza Virus Present in the Mouse Lung.* (18908)

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Previous studies(1) have indicated that when mice are inoculated intranasally with small amounts of influenza virus, death occurs only in those instances in which the concentration of virus in the lungs reaches a certain level. However, when significantly larger amounts of virus are inoculated, death may occur within 2 to 4 days, but the maximum concentration of virus attained in the lungs is usually less than the critical concentration of smaller doses and, furthermore, may be less than frequently is found in the lungs of mice that have received sublethal doses. Hence, it was suggested that when the large amount of virus was inoculated the quantity introduced may, by itself, have been sufficient to kill the animal without subsequent multiplication of the agent. As part of a further investigation of this problem, a number of experiments were conducted comparing the effect of intranasally administered immune serum on the outcome of the infection, with the concentration of virus in the lungs of the mice at the time the serum was given. The purpose of this paper is to report the results of those experiments.

Materials and methods. A mouse-adapted line of the Cam strain of influenza A virus was used. The virus had been through 128 mouse passages at the beginning of the investigation and was maintained during the period of study by bi-weekly passage in mice. At each passage a number of 9- to 12-day-old

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* This investigation was supported by a research grant from the National Institutes of Health, Public Health Service.

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embryonated eggs were inoculated intra-allantoically with a 10^{-3} dilution of the infected mouse lungs and were then incubated for approximately 48 hours at 35°C. After chilling, the allantoic fluids were harvested and were used as the source of virus; dilutions were prepared in 10% normal horse serum-distilled water. Between 0.08 and 0.1 ml of virus suspension was used as the infecting dose, and, the inoculations were made with the animals under light ether anesthesia. The mice were from a colony of albinos that had been maintained in this laboratory for over 14 years, and were approximately 4 weeks of age at the time of inoculation.

A single pool of serum, obtained from rabbits that had been immunized by 3 or 4 intraperitoneal injections of infected allantoic fluids, was used throughout. Each of the serum-treated mice received 0.06 ml of undiluted serum intranasally.

For each virus titration, 3 animals were sacrificed, the lungs pooled and ground in a mortar with alundum; sterile distilled water was added to make a 10% suspension. The suspensions were centrifuged at low speed for 5 to 10 minutes, and the supernatant fluids were removed and tested immediately. Tenfold dilutions of the fluids were prepared in infusion broth containing penicillin, and 0.1 ml of each dilution to be tested was inoculated into the allantoic sac of each of 4 to 7 (usually 5) 9- to 13-day-old fertile eggs. After 48 to 65 hours' incubation at 35°C, allantoic fluid was obtained from each egg and tested for the presence of hemagglutinins The egg-infectious titers are expressed as the reciprocal of the log of the 50% endpoint as calculated by the method of Reed and Muench(2).

Experimental. For each experiment a number of mice were inoculated intranasally with a selected virus preparation and were then divided into 2 groups. One group was held for observation as a control on the virulence of the inoculum (untreated mice), and the second group was treated with antiserum either 1 or 24 hours later in order to determine its effect upon the outcome of the infection. Comparison with the concentration of virus in the lungs of the mice was made by sacrificing 3 of the untreated animals and determining the virus titer as described under "Methods." When the serum was given 1 hour after infection, the animals to be used for the titrations were sacrificed 1 to 2 minutes following inoculation. This procedure was followed because it seemed important to allow adequate time for virus-cell combination to take place and, in addition, to base the comparison on the highest concentration of virus that had been present in the lungs; previous tests had indicated that the titer never increased but frequently decreased during the first 4 hours. When the comparisons were made 24 hours after infection, the mice were sacrificed for virus titration at the same time that the serum was given. In some experiments (Table II) additional virus titrations were made daily for a period of 3 days following the administration of serum in order to determine its effect upon the subsequent multiplication of the virus. All experiments were terminated on the 14th day.

Results. The data in Table I show that when the mice were inoculated with sufficient virus to produce immediately thereafter an egg-infectious titer of $10^{-5.6}$ or more in the lungs, the intranasal administration of immune serum 1 hour later had little or no effect in preventing the death of the mice. In contrast, when the mice were inoculated with relatively small amounts (10^{-5} dilutions) of virus and were not tested until 24 hours later, the same amount of the same serum protected either all, or the majority of the animals, even though those animals frequently had between 10 and 100 times as much virus in their lungs at the time of the administration of the serum as did the mice that had received the larger doses of virus and that were not protected. Thus, it is evident that the amount of virus required to overcome the protective effect of the immune serum was less when all of the virus present had been introduced directly into the lungs than it was when the virus present in that tissue had been produced by *in vivo* multiplication.

The data in Table II show that this dif-

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2. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

TABLE I. Comparison of Protective Effect of Intranasally Administered Immune Serum with Concentration of Virus in Lungs of Mice 1 and 24 Hr after Infection with Different Doses of Virus.*

	1 hr after infection			24 hr after infection	
Infecting dose	Egg-infectious titer of lungs†	Protection with immune serum	Infecting dose	Egg-infectious titer of lungs	Protection with immune serum
Undil.	6 ‡	0/7 §	10^{-5}	6.4‡	10/10§
"	6	0/10	"	6.6	6/7
"	5.8	0/8	"	6.7	7/7
"	5.7	1/7	"	6.8	5/8
"	5.6	2/11	"	7	7/8
10^{-1}	4.8	7/8	10^{-4}	7.8	0/7
10^{-2}	4.2	8/8	"	8.4	0/7

* The virulence of the test dose was controlled by including in each experiment a number of infected mice that were not treated with serum. All of those animals died.

† The egg-infectious titers of the lungs were determined on animals sacrificed 1 to 2 minutes after infection; other tests indicate that the values shown are as high or higher than they would have been 1 hr later at the time the serum was given.

‡ Reciprocal of the log of 50% end point.

§ No. survived/No. inoculated.

TABLE II. Effect of Immune Serum Given Intranasally After Infection Upon the Multiplication of Virus in the Mouse Lung.

Infecting dose of virus	Hr before serum was given	Exp. No.	Treatment of infected mice	Egg-infectious titer of lungs hours after serum was given 0	24	48	72	Results of infection in mice
Undil.	1	1	none	5.5*	6.5	6.5	7.2	0/10†
			serum	—	6.7	6.5	6.7	1/10
		2	none	6	6.8	6.5	7.3	0/10
			serum	—	6.5	6.5	6.3	0/10
		3	none	7	8.1	7	7.2	0/12
			serum	—	7.4	7.3	6.3	0/12
10^{-5}	24	4	none	6.3	8.2	8.3	8.5	0/10
			serum	—	2.5	7	7.3	10/10
		5	none	6.5	9.5	8.6	9	0/7
			serum	—	5.2	7.7	8	5/7
		6	none	6.7	8.3	8.3	9.5	0/11
			serum	—	7.2	7.3	8	7/11

* Reciprocal of log of 50% end point.

† No. survived/No. inoculated.

ference in the effectiveness of the immune serum was not due to the amount of infectious virus that was subsequently produced in the lungs. Although the serum seemed to suppress virus multiplication to a greater extent when given 24 hours after inoculation of small amounts of virus than when given 1 hour after inoculation of the larger amounts, it will be noted that in the latter cases all but one of the animals died, even though the maximum titers observed in 2 of the 3 groups (Experiments 1 and 2) were less than the corresponding titers found in the serum-treated groups (Experiments 4, 5, and 6) which had been previously inoculated with the smaller virus doses and in which the majority of the animals were protected. These results would seem to indicate that when very large amounts of virus are used for infection death does not depend upon the subsequent multiplication of the virus.

However, when small doses were used for infection the protective effect of the immune serum apparently was related to the concentration of virus attained in the lungs. In those tests the maximum titer observed in one of the serum-treated groups (Experiment 4) was only $10^{-7.3}$ and all of the animals survived; but in the 2 other serum-treated groups (Experiments 5 and 6) the maximum titers found were $10^{-8.0}$, and in each of those groups some, but less than half, of the animals died.

These results are in agreement with those of the previous study(1) which indicated that if small amounts (less than 100 m.l.d.'s) of virus are inoculated intranasally, a titer of approximately $10^{-8.0}$ in the lungs represents a lethal concentration. It is suggested therefore that the immune serum protected those animals in which virus multiplication was suppressed sufficiently to keep the concentration in the lungs below that critical level.

Discussion. Immune serum introduced into the lungs of mice would not be expected to have any great effect on virus multiplication in previously infected cells, but it might be expected to interfere with the spread of the virus. Hence, the difference in the effectiveness of the immune serum in the two sets of experiments reported here might be due, at least in part, to a difference in the number of cells that were infected at the time the serum was given. It is probable that when large doses of virus were inoculated a majority of the lung cells were infected immediately, but that when small doses were used only a few cells were infected at the time of inoculation, and it is also possible that in the latter instances the virus produced during the first 24 hours was confined to a relatively small area of the lungs so that the serum was present before a critical number of cells became involved.

However, if the outcome of the infection was related to the number of lung cells that became infected, injury to those cells would not seem to have always depended upon virus multiplication. The results reported here, as well as those of experiments previously reported(1,3), strongly indicate that when very large amounts of virus are used for infection the extent of lung injury may be greater but the amount of infectious virus produced considerably less than when smaller doses are used. It is of course possible that when large doses are used there is produced, in addition to the infectious virus, a considerable amount of "immature" or "incomplete" virus similar to that which has been reported to be produced under certain conditions in embryonated eggs(4,5), and also in the brains of mice following intracerebral inoculation of non-neurotropic strains(6). But if particles of that sort occur in the mouse lung only when large doses are used for infection and not when smaller doses are used, it might be because the large amount of inoculated virus is toxic to the mouse lung and injures a majority of the cells to the extent that they are no longer able to support the reproduction of complete virus. And in that event, the injury produced at the time of inoculation might be sufficient to cause the death of the animal without any subsequent multiplication of the agent.

Summary. When mice were infected by the intranasal route with large doses of influenza virus and immune serum was introduced into the lungs 1 hour later, the animals died even though neither the amount of infectious virus in the lungs at the time the serum was given nor the amount subsequently produced was as great as the corresponding amounts found in the lungs of mice that had been infected with small doses of virus and that were protected when immune serum was given 24 hours later. The findings suggest that a large amount of influenza virus introduced directly into the mouse lung may, by itself, be sufficient to kill the animal without any subsequent multiplication of the agent.

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Received July 13, 1951. P.S.E.B.M., 1951, v77.

A Simple Method for Production of DCA Hypertension in Rats. (18909)

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The chronic administration of desoxycorticosterone salts, in particular desoxycorticosterone acetate (DCA), has been employed to produce hypertension in various laboratory animals(1). It is generally agreed, however, that DCA hypertension uniformly occurs only when certain favorable conditions are present(2). In the rat these include a large dosage of DCA, a young animal, an adequate amount of available sodium, and the removal of one kidney, to "sensitize" the animal to the effects of DCA(3). Despite the presence of these suitable conditions, however, certain investigators have been unable to produce DCA hypertension of satisfactory degree or with any measure of regularity in the rat(2) or in the dog(4). We encountered similar difficulties and have attempted to introduce various modifications which might produce more regularly and frequently in our rats, significant hypertension, without marked general toxicity. The most successful method devised by us has been the following.

A one-stage operation is performed through bilateral dorsal incisions in rats of the desired age. This consists of a uninephrectomy with the application of a figure-of-eight silk ligature about the poles of the remaining kidney (Grollman)(5). When such rats are fed a stock ration, with tap water to drink, hypertension develops in from 30 to 60% of our operated animals. In an extensive experience with this technic, we have found that a systolic pressure exceeding 135 mm of Hg develops in less than 5% of rats if this level has not been reached and maintained during a 4-week postoperative period. Ordinarily, the varying number of rats in which hypertension fails to develop or to persist are discarded. In 3 independent groups of such rats we have observed that the administration of DCA in the absence of increased sodium intake in the drinking fluid, regularly has produced a significant hypertension without gross general toxicity.

A representative series of rats will illustrate this method. A group of 7-week-old male rats (Long-Evans strain) was subjected to the Grollman procedure(5). The systolic blood pressure was obtained at weekly intervals by the microphonic method of Friedman and Freed(6). During the ensuing 10-week observation period, 10 of those rats (Group I) which failed to develop persistent hypertension were maintained on the stock ration with tap water to drink. They remained normotensive, gained weight normally, and did not manifest any untoward effects or evidence of renal insufficiency. The average blood pressure at the end of this 10-week period was 119 mm of Hg. (Range: 110 to 135). These 17-week-old rats were then given 4 mg of DCA in benzyl alcohol[†], subcutaneously, 3 times weekly. A group of 8 intact, 13-week-old animals (Group II) was also injected with the same dosage of DCA and received 0.9% sodium chloride in the drinking fluid. Another group of 11 seven-week-old rats (Group III) was uninephrectomized and given 0.9% saline to drink at the same time that DCA was administered in the same dosage.

From Fig. 1 it can be seen that the average blood pressure of the rats with the "renal tie" (Group I) rose progressively and stabilized at significantly hypertensive levels

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[*] Research Fellow of the American Heart Association.

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[†] We are grateful to Dr. Abbott W. Allen of the Schering Corporation for generous supplies of DCA employed in these studies.

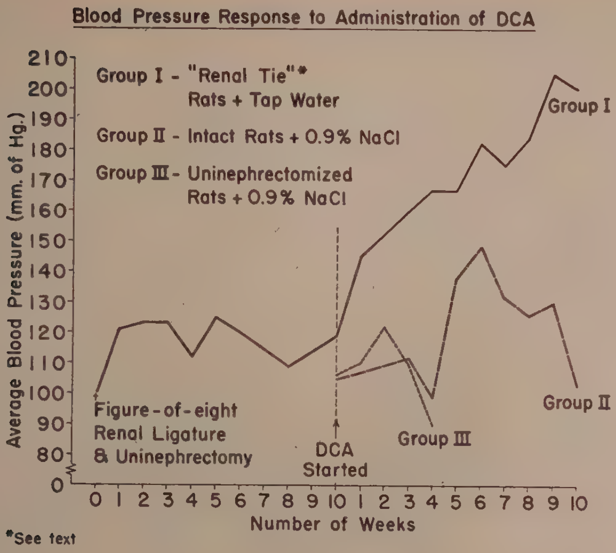

FIG. 1.

during the ensuing 11-week interval, well above the levels of the control animals. Thus, the average blood pressure of the experimental rats was 195 mm of Hg (Range: 156 to 230) at the eleventh week. Growth and weight gain were not disturbed and the general condition of these hypertensive rats remained good, with no outward evidence of toxicity. Only one rat died during the experimental period, at the ninth week, the cause of death unknown.

The average blood pressure of the intact rats receiving DCA and drinking 0.9% saline (Group II) rose from 105 mm of Hg (Range: 92 to 110) to 149 mm of Hg (Range: 120 to 180) at the sixth week. However, this response was not maintained and the average blood pressure of these rats had decreased by the tenth week to 103 mm of Hg (Range: 66 to 159). The average blood pressure of the uninephrectomized rats drinking 0.9% saline (Group III) failed to rise to hypertensive levels during the administration of DCA. Progressive loss of weight and other evidence of toxicity were observed in both groups of animals. This was so marked in the rats of Group III that the experiment was terminated.

Summary. The administration of DCA to normotensive rats following a figure-of-eight "renal tie" and uninephrectomy has been found to induce consistently a marked hypertension. Similar treatment of both intact and uninephrectomized rats drinking physiologic saline was ineffective in this respect. Hypertension was readily induced in the "renal tie" animals despite the absence of excess sodium chloride and their older age.

Received July 15, 1951. P.S.E.B.M., 1951, v77.

Beta-Glucuronidase in Leukocytes, Plasma, and Urine of Patients with Nephrosis Undergoing Treatment with ACTH.* (18910)

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(Introduced by E. A. Zeller.)

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B-glucuronidase has been associated with the metabolism of glucuronic acid, of the estrogens, and of alkaloid poisons such as borneol. It has also been shown that this enzyme's activity is increased in malignant tissue and that cortical steroids are excreted for the most part as glucuronides. Fishman(1) showed that activity of mouse liver was increased following administration of borneol and menthol which are conjugated by the animal into the glucuronide form. Later he showed that mouse uterus activity is increased by administration of estrogens thus suggesting the possibility of an active conjugated form of the estrogen(2). Odell and Fishman(3) have shown that the endometrium has cyclic changes paralleling that of the menstrual cycle. Blood and urine glucuronidase have been found to be increasingly elevated during pregnancy(4). Fishman noted an elevation of B-glucuronidase activity in carcinomas of various organs(5) and recently there has been an attempt made to develop a "limited selective test for cervical (cancer) suspects"(6) by determining the B-glucuronidase activity of vaginal fluid. It has been also shown that the corticosteroids in human urine are for the most part present as glucuronic acid conjugates(7). Although these many facts about the enzyme have been ascertained, its physiological significance is not yet absolutely clear since the precise mode of action of the enzyme *in vivo* is still somewhat obscure.

We have employed in our laboratory for a considerable time the glucuronidase hydrolysis method for the measurement of the corticosteroids excreted in the urine of patients before, during, and after administration of adrenocorticotropin (Armour) in order to assay the degree of cortical activity elicited by the ACTH and to compare this with clinical response. In addition, from time to time, we have determined the free corticosteroids present in the urine. From this we have seen that favorable clinical response does not absolutely follow an increased cortical output (although in the cases of striking improvement a potent activation was achieved) and have also noted that the ratio between the free and total corticosteroids does not remain constant during ACTH therapy. Since the cortical hormones are demonstrably associated with glucuronic acid and since glucuronidase, capable of either hydrolysis or conjugation of the hormones, might be responsible for the difference in clinical response by reason of its supplying an abundance of the active form, it was decided to investigate the B-glucuronidase activity of blood and urine.

Methods. Glucuronidase assay: The beta-glucuronidase was assayed by the method of Fishman, Springer, and Brunetti(8). The method was found to give very reliable duplicates. The transmittance readings agreed within 1%. Control tubes were prepared containing (1) substrate and buffer (2) sample without substrate. The results were calculated as averages of the duplicates. According to the recommendations of Fishman,

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* This investigation was supported by a research grant from the National Heart Institute, of the National Institutes of Health, Public Health Service.

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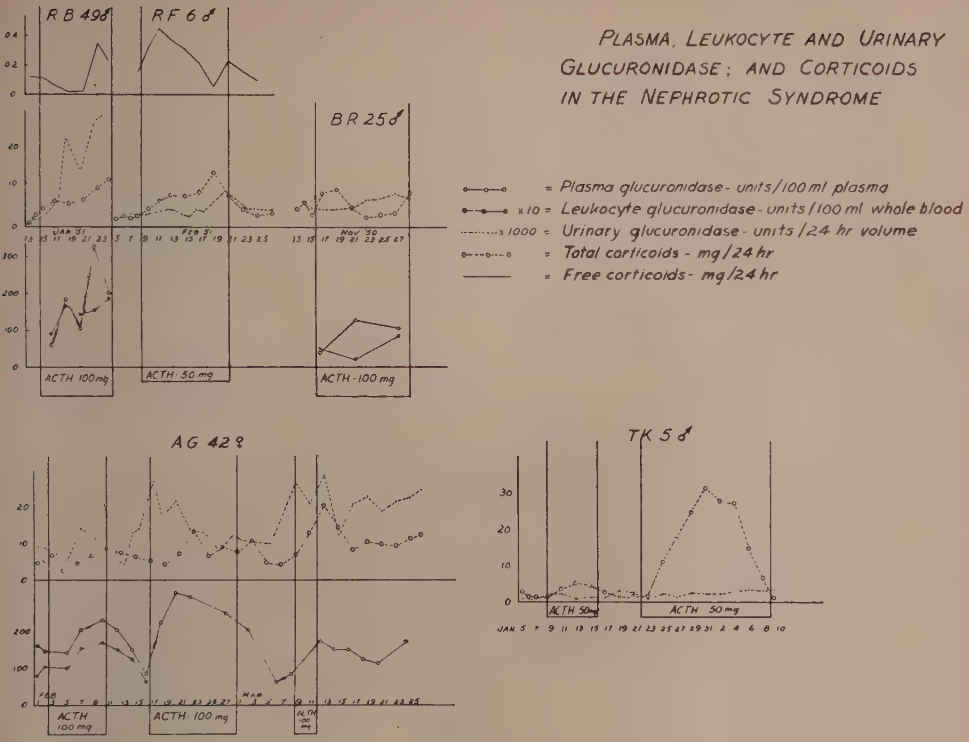

FIG. 1.

the urine samples were not subjected to dialysis. One enzyme unit refers to the liberation of 1 γ per hour of phenolphthalein. 0.1 cc of urine was used with the recommended buffers and substrate and incubated at 37.5°C for 10-20 hours.

Free Corticosteroid Assay: Free urinary corticosteroids were determined by analyzing the fresh, 24 hour urine without acidification and without enzymatic hydrolysis.

Total Corticosteroid Assay: Total urinary corticosteroids were obtained by hydrolyzing 25 ml aliquots of 24 hour urines with 7,500 Fishman units of B-glucuronidase for 48 hours at 37°C, after adjusting to pH 4.5 and adding 2.5 ml of 1-M acetate buffer. The hydrolyzed urine was then acidified, extracted, and analyzed by the periodic acid method(9-11).

The cases studied represent 4 uncomplicated nephrosis patients and one recurrent nephritis patient. A. G. is a 42-year-old female, R B., a 49-year-old male, R. F., a 6-year-old male, T. K., a 5-year-old male, and B. R., the recurrent nephritic, is a 25-year-old male.

Results. Fig. 1 shows the glucuronidase activity of plasma (5 courses of treatment), leukocytes (3 courses), and urine (7 courses). In addition are given the data for the corticosteroids (total) in all the courses and the free corticosteroids in 2 of the courses of ACTH.

Plasma Glucuronidase: In all 5 courses studied for plasma glucuronidase activity, we are able to show a marked rise in the plasma glucuronidase level. In all cases, there is seen a distinct correlation between the plasma glucuronidase and the total urinary corticosteroid level. In the one case where the

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10. Cohen, S. L., *J. Biol. Chem.*, 1950, v184, 417.

11. Corcoran, A. C., Personal communication.

free corticosteroids are shown with the plasma glucuronidase, the plasma glucuronidase parallels this closely. The plasma activity, as well as the leukocyte activity, tends to sustain itself for several days after withdrawal of the ACTH.

Leukocyte Glucuronidase: In 2 of the 3 courses of ACTH studied, the white blood cell activity increases almost exactly proportionally to the rise in the plasma glucuronidase. In the third there is a temporary decrease followed by an increase in the enzyme activity.

Urinary Glucuronidase: Five of the 7 courses of ACTH on 5 patients show a definite rise in urinary B-glucuronidase. Two show no significant response. Both of these cases are children under 7 years of age. It cannot be said that there is any strict correlation between the urinary glucuronidase and the total corticosteroids. The corticosteroids increase rather uniformly during the treatment whereas the B-glucuronidase shows marked fluctuation. Nor can any correlation be discovered between the enzyme activity and the free corticoids as determined in 2 of the cases.

Total and Free Corticosteroids: All of the patients showed a rather uniform rise in total corticosteroids excreted during ACTH administration. The response on the peak day of corticosteroid output as compared with the control days varied individually from a 3-fold increase to a 15-fold increase.

In regard to the free corticosteroids as studied in 2 of the cases a little more can be said. In R. B. the clinical response was meager on the whole. However, the greatest decrease in cholesterol occurred during the time that the free corticosteroid level was at its peak. There was no appreciable diuresis in this case. In R. F. the marked increase in the free corticosteroid (5-fold increase) was accompanied by a considerable decrease in cholesterol and a major diuresis. In this respect the number of cases limits absolute correlation, but it can be said that the peak in free corticosteroid level occurred at the time of the most vigorous clinical response.

Discussion. Plasma and leukocyte B-glucuronidase values are subject to individual variation and since there are not, to our knowledge, any normal urinary values available, we are not able to say whether or not these fall within the normal range. However, on the days prior to ACTH administration the values obtained in the glucuronidase activity were fairly constant per individual.

The results seem to indicate that the action of the ACTH on the B-glucuronidase is a secondary one, contingent upon the increased output of the cortical hormones. If the action were a direct one it would be expected that the B-glucuronidase would increase before the corticosteroid rise whereas we found that the plasma and urinary glucuronidase increase paralleled or followed the corticosteroid increase.

From the data presented there is no evidence for believing that the B-glucuronidase mechanism accounts for the difference in clinical response and cortical response of a patient. Good response in the case of B. R. was accompanied by increased plasma and urinary enzyme activity while the relatively poor response in the case of R. B. resulted in a similar rise in the enzyme activity.

Summary and Conclusions. 1. Observations on the effects of activation of the adrenal cortex by ACTH on the B-glucuronidase activity of plasma, leukocytes, and urine are reported. 2. ACTH administration evokes a marked rise in plasma and white cell glucuronidase activity. Plasma glucuronidase activity correlates well with the total corticosteroid level elicited by the ACTH in that it increases during administration and decreases following administration although the day-to-day parallel is variable. 3. Urinary glucuronidase activity fails to indicate significant correlations either with plasma and white cell glucuronidase or with the corticosteroids.

We wish to extend our thanks to Dr. E. Albert Zeller for his helpful advice in the technical details of the work and in the preparation of the paper.

Received July 16, 1951. P.S.E.B.M., 1951, v77.

A Rapid Method for the Determination of Ultramicro Quantities of Calcium and Magnesium.* (18911)

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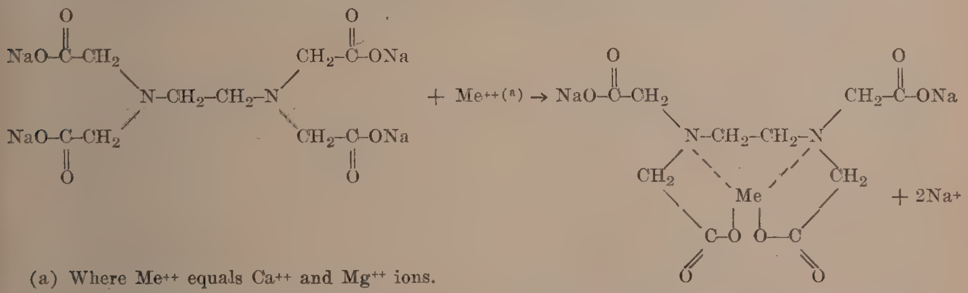
This paper presents a rapid method for the estimation of calcium and magnesium on small volumes of serum (20 to 50 μl) representing 0.05 to 0.175 micromole. Whereas by means of the flame photometer rapid estimation of serum sodium and potassium is possible (and often plays an important part in saving the lives of patients), the hitherto available methods for serum calcium require at least 3 to 4 hours and sometimes longer. This period of time is too long when it becomes necessary to decide whether low serum calcium is a factor in the cause of convulsions. Management of such patients thus usually begins without the benefit of objective evidence.

Since this problem is often encountered in newborns and prematures, a rapid method which requires only small amounts of serum is of greater usefulness. This is especially so when multiple analyses such as pH, CO_2, sugar, K, Na, and Cl are involved.

The present method in principle is an ultramicro modification of the method of Schwarzenbach and Ackerman(1) proposed for the determination of water hardness. Alkaline earth metals (calcium and magnesium) form tightly bound chelate complexes with ethylene-diamine-tetraacetate as shown below:



(a) Where Me^{++} equals Ca^{++} and Mg^{++} ions.

The chelation occurs quantitatively at a pH of 10.0 to 10.5. The dye, eriochrome black T, changes from a wine-red in the presence of free alkaline earth metallic ions to a blue-green in their absence. The proposed buffer of ammonium chloride-ammonium hydroxide(1) was replaced by the more stable ethanolamine which proved to be not only a stable buffer, but under the conditions employed provides essentially the same pH when added to serum as with the pure inorganic solutions of calcium and magnesium. Fortunately the titration can be carried out in serum itself, the color of the end point being blue-green instead of the blue of pure inorganic solutions. This blue-green is due to the coloration of the serum, which is normally yellow and which when combined with the blue of the end-point gives a blue-green. To aid in obtaining the end-point, a match color titration is employed. To a sample of serum, an excess of disodium ethylene-diamine-tetraacetate (SED) is added containing the same proportions of buffer and eriochrome black T as is employed in the method. The titration represents the sum of serum calcium and magnesium. For rapid and approximate calcium values, the titrimetric equivalent of the mean magnesium value found in human serum is subtracted from the total titration, and as seen in Table I one obtains results that will guide the clinician in his treatment. The combined value, how-

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* This investigation was supported by the research grant from the National Institute for Dental Research of the National Institute of Health, Public Health Service.

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TABLE I. Approximate and Rapid Estimation of Serum Ca (.05 ml serum). All values are expressed as milliequivalents per liter of serum.

Patient	Col. 1 Total Ca + Mg	Col. 2 Approximate Ca	Col. 3 Actual Ca	Col. 4 Difference
1	6.95	5.31	4.63	+.68
2	6.46	4.82	4.83	—.01
3	6.44	4.80	4.77	+.03
4	6.20	4.56	4.63	—.07
5	5.96	4.32	4.58	—.26
6	5.95	4.31	4.66	—.35
7	5.94	4.30	4.19	+.11
8	5.85	4.21	4.38	—.17
9	5.82	4.18	3.99	+.19
10*	5.63	3.99	3.94	+.05
11*	4.80	3.16	3.04	+.12
12*	4.68	3.04	2.98	+.06

* Sera No. 10, 11, 12 were emergency determinations in which the patients were in convulsions. The rapid estimation was followed promptly by calcium salt administration to alleviate the convulsions.

Col. 2—Obtained by subtraction of 1.64 mE/L of magnesium as the mean magnesium content in human sera from total mE/L (Ca + Mg) in Col. 1. Col. 3—Determined by the method of Sobel *et al.*(6,7). Col. 4—Difference between Col. 2 and Col. 3.

TABLE II. Exact Estimation of Serum Calcium and Magnesium. All values expressed as milliequivalents per liter of serum.

	Analytical data			Calculated data			
Patient	Col. 1 New method Ca + Mg	Col. 2 Ca	Col. 3 Mg	Col. 4 Ca	Col. 5 Difference	Col. 6 Mg	Col. 7 Difference
1	6.50	4.78	1.69	4.81	+.03	1.72	+.03
2	6.44	4.77	1.65	4.79	+.02	1.67	+.02
3	6.35	4.57	1.89	4.46	—.11	1.78	—.11
4	6.04	4.48	1.65	4.39	—.09	1.56	—.09
5	5.96	4.77	1.32	4.64	—.13	1.19	—.13
6	5.91	4.57	1.36	4.55	—.02	1.34	—.02
7	5.78	4.57	1.36	4.42	—.15	1.21	—.15
8	5.78	4.08	1.77	4.01	—.07	1.70	—.07
9	5.64	4.24	1.40	4.24	.00	1.40	.00
10	5.54	3.84	1.61	3.93	+.09	1.70	+.09

Col. 2—Determined by method of Sobel *et al.*(6,7). Col. 3—Determined by method of Orange and Rhine(5). Col. 4—Obtained by subtraction of Col. 3 from Col. 1. Col. 5—Difference between Col. 2 and Col. 4. Col. 6—Obtained by subtraction of Col. 2 from Col. 1. Col. 7—Difference between Col. 3 and Col. 6.

ever, may turn out to be more significant in convulsions than the evaluation of serum calcium alone, since convulsions have been observed due to low serum magnesium alone(2-4).

For precise calcium values, magnesium can be determined by the method of Orange and Rhine(5) which in itself requires only half an hour to complete, and as seen in Table II, the calcium values thus obtained compare favorably with those obtained by calcium oxalate precipitation(6,7). For precise magnesium values, the titrimetric equivalent of calcium is determined in the usual way(6,7) on 50 to 100 microliters of serum and the value subtracted from the total titration, and as seen in Table II, magnesium values compare favorably with those obtained by the method of Orange and Rhine(5).

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Present experiments in progress indicate that it will be possible by using murexide as an indicator to develop a direct titration for calcium and by using oxalate or fluoride to tie up the calcium and to titrate magnesium directly. Moreover, the precipitated calcium oxalate can be titrated by SED. Serum carbonate (CO_2) can be determined by precipitating as barium carbonate and titrating the barium with SED. Similarly a rapid titrimetric method for sulfates is possible involving precipitation of the sulfate as the barium salt and titration of the barium with SED. Thus it appears that SED titration may become a valuable adjunct to clinical chemistry especially when ultramicro quantities are involved.

Experimental. Reagents and Apparatus—

1. 0.17M Ethanolamine (Approximate) 10.2 ml of ethanolamine is diluted to 1000 ml with doubly distilled water (Eastman-Kodak grade).

2. Disodium ethylene-diamine-tetraacetate (SED)—Dissolve approximately 4.5 g of the salt in 1000 ml of doubly distilled water (Fisher analytical grade). This solution is standardized against a known calcium solution and is stable for at least one month.

3. Stock Calcium Solution—1 ml is equivalent to 0.200mM Ca^{++}. Dissolve 2.0018 g dried $CaCO_3$ in a minimal amount of 6N HCl. Evaporate to dryness on a steam bath and continue to heat until no more fumes of hydrochloric acid are evolved. Dissolve the remaining solid in 100 ml of doubly distilled water.

4. Standard Calcium Solution—1 ml is equivalent to 2.00 micromoles or 4.00 meq/L Ca^{++}. Dilute 1 ml of stock calcium solution to 100 ml with doubly distilled water.

5. Stock Magnesium Solution—1 ml is equivalent to 0.200 mM Mg^{++}. Dissolve 4.9087 g of dried $MgNH_4PO_4 \cdot 6H_2O$ in 100 ml of 0.1 N HCl.

6. Standard Magnesium Solution—1 ml is equivalent to 1.00 micromoles or 2.00 meq/L of Mg^{++}. Dilute 1 ml of stock magnesium solution to 200 ml with doubly distilled water.

7. Eriochrome black T indicator solution—Dissolve 2 g of the solid dye in 500 ml of methyl alcohol and add 20 ml of concentrated ammonium hydroxide.

8. Mixed buffer indicator solution—This solution must be prepared freshly before use. It is stable for approximately 3 hours. To every 5 ml of ethanolamine buffer add 2 drops of the indicator solution. The color of the resulting solution should be blue. If there is any trace of red or purple add trace amounts of SED until color of the solution is blue.

9. Microburette—The Gilmont ultramicro burette was used in this study. The total capacity of this burette is 100 microliters. The total volume can be delivered in steps of 1/1000 of the total so that each scale is 0.1 microliter and 0.01 microliter may be estimated with a fair degree of accuracy. The stirring device of the manufacturer was discarded and in its place a Spinal (Quincke) B-D Yale Luer-Lok, 20 x 3½ inch hypodermic needle was used. The needle point was ground flat and the Luer-Lok end was ground so as to form a round cylinder instead of the normal 4-sided appearance. This modification allows the air stream that is used for agitation during titration to be introduced to the very bottom of the titrating tube.

10. Ultramicro pipettes—Kirk(8,9) type ultramicro pipettes were used in this study. These pipettes are calibrated to contain, and in use the contents are washed into the titration medium and the pipettes are washed by drawing the medium up into and blowing out the pipette. All the washings are added to the titration medium.

11. Small test tubes—75 x 10 mm. These are standard agglutination tubes.

Estimation of total serum calcium and magnesium. With a 20 or 50 microliter pipette transfer a sample of serum to a test tube containing 0.5 ml of the mixed buffer plus indicator solution. The pipette is rinsed by alternately sucking and blowing the contents of the test tube 3 times. Owing to the 10 to 20 fold dilution of the serum with the buffer-indicator mixture, the loss due to the film re-

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8. Kirk, P. L., and Craig, R., *J. Lab. and Clin. Med.*, 1933, v18, 81.

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maining in the pipette is negligible. Titrate the solution with SED contained in the microburette to the blue-green endpoint of a sample of serum plus buffer-indicator mixture that has had an excess of SED added to it. This titration represents the sum of calcium and magnesium. For a normal serum the titration will be approximately 14.0 microliters (which represents 140 full scales on burette) for 50 microliters of serum and 5.6 microliters for 20 microliters of serum.

At the same time, the SED is standardized by transferring 20 or 50 microliters of the standard calcium solution to a test tube containing 0.5 ml of buffer-indicator solution and the solution is titrated with SED to the blue endpoint of the pure buffer-indicator solution. The value obtained is the standardization of the SED. The normality of the SED is then calculated in the usual fashion. For the sake of convenience, millinormality is employed (mE/L) which is obtained by multiplying normality by 1000.

For equal volumes of standards and sera, the total mE/L of calcium plus magnesium in the sera are obtained as follows:

$$\text{mE/L (Ca + Mg)} = \frac{\text{Titration of serum}}{\text{Titration of Ca standard}} \times 4$$

(Table I, Col. 1)

To obtain the approximate calcium content of the serum, a mean value for magnesium of 1.64 mE/L (2.0 mg %) is assumed:

$$\text{mE/L Ca} = \text{mE/L (Ca + Mg)} - 1.64$$

(See Table I: Col. 3 = Col. 1—1.64)

Exact determination of serum calcium. Magnesium is determined on a 0.1 ml aliquot of serum using the method of Orange and Rhein(5). For equal volumes of standards and sera the exact calcium content is determined as follows:

$$\text{mE/L Mg} = \frac{\text{mg \% Mg} \times 10}{12.16}$$

$$\text{mE/L Ca} = \text{mE/L (Ca + Mg)} - \text{mE/L Mg}$$

(See Table II: Col. 4 = Col. 1—Col. 3

Determination of exact serum magnesium. Serum magnesium may be determined exactly by this method if an aliquot of the serum is analyzed for calcium by the method of Sobel *et al.*(6,7). For equal volumes of standards and sera, the exact magnesium value is determined as follows:

$$\text{mE/L Ca} = \frac{\text{mg \% Ca} \times 10}{20.04}$$

$$\text{mE/L Mg} = \text{mE/L (Ca + Mg)} - \text{mE/L Ca}$$

(See Table II: Col. 6 = Col. 1—Col. 2)

Interferences. Iron will interfere with the SED titration resulting in high positive errors. Hemolyzed sera therefore cannot be used with this method. Moreover, since red blood cells contain a higher percent of magnesium than serum, hemolyzed sera will have positive errors due to magnesium when titrated with SED.

Summary. A method is presented for the ultramicro estimation of serum calcium and magnesium with ethylene-diamine-tetraacetate in the presence of eriochrome black T, at a pH of 10.0 to 10.5. The serum (0.02 to 0.05 ml) is titrated directly and the titration is completed in less than one minute; thus the speed of this determination compares favorably with that of the flame photometer. For rapid and approximate calcium value, the mean magnesium concentration of human serum (1.64 mE/L) is subtracted from the total calcium plus magnesium thus determined. For precise calcium values, magnesium is determined on an aliquot of serum and the value subtracted from the calcium plus magnesium values. For precise magnesium values, calcium is determined on an aliquot of serum and the value subtracted from the calcium plus magnesium values.

Received July 16, 1951. P.S.E.B.M., 1951, v77.

Effectiveness of Procaine Amide in Digitalis-Induced Ventricular Tachycardia.* (18912)

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Procaine amide (Pronestyl, Squibb) has been shown to be effective in the treatment of ventricular arrythmias(1-3). The pharmacology of procaine amide and its physiological disposition has recently been reported(1,4,5). The present report was designed to determine the value and limitations of procaine amide in the treatment of ventricular tachycardia produced acutely by large intravenous doses of cardiac glycosides.

Experimental. Twenty mongrel dogs weighing between 5 and 13 kg were anesthetized with 50 mg/kg (i.v.) of Dial-Urethane. Supplemental pentobarbital was administered in a few cases to induce an adequate level of anesthesia. Thirteen of the dogs were subjected to bilateral cervical vagosympathectomy; final analysis, however, indicated no differences in the procaine amide action in the vagotomized and non-vagotomized animals. Seven of the vagotomized animals were artificially respired with oxygen at rates of 16 to 20 per minute by means of a rhythmic interrupter interposed between the animals and a constant pressure tank. Frequent electrocardiograms were taken in all experiments from standard Lead II with a direct writing E.P.L. Cardiotron. Digitoxin (Digitaline Nativelle, Varick) was administered to 12 dogs and Ouabain (Penick, U.S.P.) to 8 dogs. The glycosides were administered intravenously in divided doses until either A-V block with a slow ventricular rate or ventricular tachycardia had developed. The dose of glycoside necessary to produce such effects ranged from 0.75 to 1.25 cat units/kg administered within a period of 2 hours. Procaine amide in a 10% solution was administered by rapid intravenous injection in doses of 10 or 20 mg/kg to those animals in ventricular tachycardia and was repeated when necessary. In most instances, the drug was not injected until at least 10 minutes of tachycardia with no appearance of normal sinus complexes was recorded. This procedure was intended to minimize the possibility of spontaneous remissions of the arrhythmias. Such requirements necessitated relatively high doses of cardiac glycosides and resulted, in most instances, in tachycardia of multifocal origins. The criteria for reversion of the tachycardia, arbitrarily adopted, was a period of at least 2 minutes of ectopic-free, normal sinus rhythm.

Blood pressures were recorded in 12 of the 20 experiments. In 7 cases, the recordings were made by means of a standard mercury manometer and in 5 cases by a Statham Transducer in conjunction with a Brush strain analyzer (Model B1-310) and oscillograph (Model B1-202).

In addition to the dog experiments, the effect of procaine amide on the "cat-unit" of ouabain (Hatcher-Brody method) was determined in 7 cats. End points were obtained electrocardiographically. The procaine amide was administered by continuous intravenous infusion in 5 experiments and by rapid intravenous injections in the others. A similar "cat-unit" study was carried out with procaine and other drugs by Emerson(6).

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* This investigation was supported by a research grant from the National Institute of Health, U. S. Public Health Service.

† Fellow of the American Foundation for Pharmaceutical Education.

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TABLE I.

Exp No.	Glycoside and dose in cat unit/kg*	Initial dose of procaine amide in mg/kg and No. of init. inj	Results following initial dose of procaine amide	Additional injections	Total dose of procaine amide, mg/kg	Time of final ecg in hr†
3‡§	Digitoxin 1.25	10 (1)	Reversion to NSR‖	5	80	1
5‡§	Digitoxin 1	10 (1)	” ” ”	7	120	11+
6‡§	Digitoxin 1	10 (2) 20 (1)	” ” ”	5	100	12+
7	Digitoxin .75	10 (1)	” ” ”	0	10	12+
11	Ouabain .75	10 (2)	” ” ”	4	60	12+
15	Ouabain .75	10 (1)	” ” ”	0	10	12+
16	Ouabain .75	10 (5)	Coupling after second inj; NSR after fifth injection	0	50	12+
1‡	Digitoxin 1.25	10 (1)	Reversion to NSR	1	20	1
13	Ouabain 1	10 (2)	Coupling after first inj; complete A-V block after second inj; then cardiac arrest	0	20	—
12	Ouabain .75	10 (1)	Complete A-V block; then 8-min period of cardiac arrest	7	80	12+
2‡	Digitoxin 1	10 (1)	Complete A-V block	4	60	12+
4‡	Digitoxin 1	10 (2)	Slow idioventricular rhythm with no auricular activity	3	50	3+
17‡	Digitoxin 1	10 (1)	Cardiac arrest	0	10	—
14	Ouabain 1	10 (1)	Complete A-V block; then cardiac arrest	0	10	—

* .33 mg digitoxin = 1 cat unit; .11 mg ouabain = 1 cat unit.

† Time in hours after first procaine amide injection; in all instances, normal sinus rhythm was recorded at these times.

‡ Vagotomy.

§ Artificial respiration.

‖ NSR = normal sinus rhythm.

Results. The results of procaine amide administration to 14 dogs in digitalis-induced ventricular tachycardia are presented in Table I. Procaine amide was not administered to 6 other digitalis-treated dogs because of the development of slow idioventricular rhythms in 4 and fatal ventricular fibrillation in 2 instances.

Reversion to normal sinus rhythm. In 8 of the 14 experiments ventricular tachycardia reverted to normal sinus rhythm following procaine amide. In 5 of these, a single procaine amide injection produced initial reversion; in the other experiments additional injections were necessary. The reverted rhythm remained ectopic-free in 2 experiments; in the remaining, the initial reversion was temporary, lasting on an average of 10 minutes before the reappearance of ventricular tachycardia or the appearance of numerous premature ventricular contractions. The recurring arrhythmias were generally not as severe as the previous tachycardia, being usually from a single focus and mixed with normal complexes. Procaine amide, administered at the appearance of these and subsequently occurring arrhythmias, was effective in re-establishing normal sinus rhythm. The incidence of ectopic rhythms progressively decreased during the course of these experiments and in all cases were absent during observations over at least a 60-minute interval before termination of the experiment. This diminishing incidence of arrhythmias was probably the result of decreasing cardiac glycoside intoxication and cumulative effectiveness of procaine amide.

In all of these experiments, a prolonged P-R interval was recorded following the initial reversion. This change was probably due to the action of the cardiac glycosides since subsequent injections of procaine amide did not appreciably lengthen the interval and also because, in most experiments, a prolonged P-R interval was recorded before the onset of ventricular tachycardia. No significant ECG changes which could be directly attributed to cumulative procaine amide toxicity were seen in those experiments with numerous administrations. Transient bundle branch block, however, occurred in 4 experiments following injection of procaine amide. This change commonly reappeared with succeeding administrations in the same experiment. Similar bundle branch block was seen after procaine amide therapy in humans by Kinsman *et al.*(2).

Ventricular fibrillation occurred suddenly in one experiment (No. 5) after procaine amide had reverted 3 episodes of ventricular tachycardia to normal sinus rhythm. The fibrillation was coarse in character and was followed by a 4-minute period of asystole, after which time the heart resumed beating in ventricular tachycardia. This tachycardia then reverted to normal sinus rhythm following an injection of procaine amide. Subsequent appearances of ventricular premature contractions were treated with 5 additional injections of procaine amide during the remainder of this experiment. As has been pointed out, ventricular fibrillation also occurred in 2 other experiments before any administration of procaine amide.

Development of Slow Idioventricular Rhythms and Cardiac Arrest After Procaine Amide Administration. In 6 of the 14 experiments with digitalis-induced tachycardia, procaine amide administration was followed by slow idioventricular rhythms and cardiac arrest. In 3 experiments (Nos. 12, 17, and 14) cardiac arrest followed shortly after a first procaine amide injection. In 2 of these (Nos. 12 and 14) a period of slow idioventricular rhythm associated with complete A-V block was recorded before the asystole. In 2 (Nos. 14 and 17), the arrest was permanent; in the other (No. 12), the heart resumed beating in normal sinus rhythm after 8 minutes. In this latter experiment, ventricular tachycardia recurred and was subsequently reverted to normal sinus rhythm by procaine amide with no further occurrences of A-V dissociation or of cardiac arrest. In Experiment No. 13 cardiac arrest followed a second injection of procaine amide. The initial injection had converted a multifocal ventricular tachycardia to coupling. Procaine amide administration, repeated for this latter arrhythmia, resulted in the development of second degree A-V heart block with infrequent ventricular responses and then permanent ventricular arrest.

In 2 experiments (Nos. 2 and 4), idioventricular rhythms, without the development of cardiac arrest, occurred after procaine amide administration. In No. 2, the idioventricular rhythm was associated with complete A-V heart block; in No. 4, there was no evidence of auricular activity associated with the idioventricular rhythm. Cutting the vagi in the latter experiment resulted in a slight increase in ventricular rate, but did not otherwise alter the conditions. Additional administrations of procaine amide in each of these experiments at the reappearance of ventricular tachycardia restored the previous forms of idioventricular rhythms without the occurrence of cardiac arrest. In both experiments, the electrocardiographic picture improved and a period of normal sinus rhythm was recorded before termination.

Effects of Procaine Amide on Blood Pres-

sure. The blood pressure usually fell about 10 to 20 mm Hg following doses of 10 mg/kg and about 30-40 mm Hg following doses of 20 mg/kg. Such hypotensive effects were, in most instances, of short duration and were occasionally followed by slight hypertensive effects when a tachycardia was reverted.

Effect of Procaine Amide on the "Cat Unit" of Ouabain. Procaine amide failed to increase the lethal dose of ouabain in cats. In 5 experiments, the drug was infused concomitantly with the cardiac glycoside at rates of from 2 mg/cc/min. (total dose of 23 mg/kg) to 20 mg/cc/min. (total dose of 157 mg/kg). In 2 other experiments, procaine amide was injected repeatedly in a 10% solution in doses of 10 and 20 mg/kg (total doses of 70 and 80 mg/kg) beginning with the first appearance of ventricular tachycardia and continuing until the end of the experiment. The ouabain was constantly infused as in the other cat experiments. The "cat unit" values, as determined with ouabain and procaine amide, were compared with the values obtained with a previously conducted series of 11 cats in which ouabain alone was used. The value for the ouabain-procaine amide "cat unit" as well as the standard deviation was the same as that obtained with the ouabain series (0.11 mg/kg SD±0.015).

Summary. Rapid intravenous administration of procaine amide was found effective in reverting ventricular tachycardia, produced in dogs by large doses of cardiac glycosides, to normal sinus rhythm. In most instances, the reversion was temporary and additional injections of procaine amide were necessary in order to maintain the normal rhythm. Such additional injections, repeated several times in these experiments, did not produce electrocardiographic signs of cumulative toxicity.

Although procaine amide effectively reverted ventricular tachycardia in the dog experiments, the same agent failed to increase the lethal dose as determined by limited "cat unit" studies. A similar relation was noted by Emerson in studies on procaine(6) and by Stanbury and Farah(7) in studies on magnesium.

The development of slow idioventricular rhythms and cardiac arrest in several experiments following procaine amide administration demonstrates a danger of drug termination of digitalis-induced ventricular tachycardia. As Gold(8) has emphasized, complete A-V heart block concomitantly produced by the digitalis may be masked in the electrocardiogram by the ventricular tachycardia and the abolition of the latter arrythmia by quinidine or other drugs may result in ventricular arrest.

We are obliged to Dr. P. C. Gazes for his review of the electrocardiographic interpretations.

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Received July 17, 1951. P.S.E.B.M., 1951, v77.

Effect of Vitamin B_{12}, Folic Acid, and Nicotinamide on Urinary Coproporphyrin Excretion in Photosensitized Rabbits.* (18913)

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Nicotinic acid has been claimed to cause a decrease in the urinary coproporphyrin in certain pathologic states. Thus Spies and co-workers reported marked diminution in patients with pellagra(1) and in painters(2).

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[*] Aided by Research Grant No. 345, U. S. Public Health Service.

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The method(3) employed, however, was subsequently shown to be nonspecific and correlated much more with urorosein than with porphyrin(3-7).

Boulin *et al.*(8) administered nicotinamide (0.5 g orally and 0.4 g intramuscularly per day) for 8 days to a patient with coproporphyrinuria and a history of alcoholism. A decrease from 646 mg in 24 hours to a normal level was reported. The same investigators studied a case of porphyria before and after administration of nicotinamide (0.5 g orally per day) and noted a decrease in the amount of uroporphyrin excreted from 2.88 mg to 1.056 in 24 hours. No definite clinical improvement was observed. The decreases in both of these instances are of doubtful significance because of the well known spontaneous fluctuations in porphyrin excretion in both conditions. A somewhat more convincing effect was described by Scolari(9) in several individuals receiving antiluetic arsenical therapy. Here it appeared that significant decreases in urinary coproporphyrin, primarily elevated following the arsenic, resulted from the administration of nicotinamide.

A relation of folic acid to porphyrin metabolism has been suggested by several investigators. Steinkamp *et al.*(10) reported an increase in urinary coproporphyrin in cases of pernicious anemia after folic acid therapy. This report, however, was not based on a quantitative procedure. Totter and coworkers(11), studying the influence of folic acid on glycine and porphyrin metabolism in rats, concluded that the folic acid significantly increased the fecal and probably the urinary porphyrin excretion of animals fed a purified diet. Although of fundamental interest, it should be noted that the principal porphyrin of rat feces is protoporphyrin, derived from the Harderian glands(12).

Kvasnickova(13), administered 15 mg of folic acid daily to a patient suffering from a cutaneous form of porphyria and who was excreting 200 mg each of uroporphyrin and coproporphyrin per liter of urine. She reported it to be highly effective; it was stated that the skin manifestations disappeared, the pigmentation cleared up and the general feeling of well-being improved; the uroporphyrin disappeared from the urine entirely. Castex, Lopez Garcia and others(14) observed two cases of porphyria, one of abdominal and another of cutaneous type, in which there was said to be a complete return of urinary coproporphyrin excretion to the normal level after the administration of 20 mg of folic acid daily. With this a complete disappearance of clinical symptoms was also noted. However, serial data were not given, and the possibility of spontaneous remissions was not excluded.

In a previous paper(15) it was shown that the photodynamic effect of Rose Bengal and ultraviolet light produces a marked increase in urinary coproporphyrin in rabbits within 24-48 hours. The purpose of the present investigation was to study the effect of folic acid, nicotinamide and vitamin B_{12} on the urinary coproporphyrin excretion under these circumstances.

Methods. Adult rabbits of either sex were

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TABLE I. Effect of Folic Acid (F.A.), Vitamin B_{12}, and Nicotinamide (N.A.) on Coproporphyrinuria Following Rose Bengal and Ultraviolet Irradiation.

No. rabbits	Coproporphyrin, μg per day				Substance administered	
	Day 0*	1	2	3		
5	7	163	72	22		
1	7	48	9	9	F.A.	5 mg
1	6	45	34	34	F.A.	15 mg
3	7	10	8	8	F.A.	45 mg
1	5	15	5	13	B_{12}†	5 μg
2	7	12	11	10	B_{12}	10 μg
2	9	6	27	13	B_{12}	15 μg
1	3	6	19	4	B_{12}	30 μg
2	9	18	17	15	Cryst. B_{12}‡	15 μg
3	12	46	60	23	N.A.	50 mg
1	9	11	209	20	N.A.	200 mg
2	8	28	64	33	N.A.	300 mg
3	6	22	19	8	F.A. B_{12}‡	5 mg 5 μg

* A control analysis of urinary coproporphyrin excretion was carried out on the day preceding the administration of Rose Bengal and ultraviolet light. Mean values are given where more than 1 rabbit was employed.

† Rubramin (Squibb).

‡ Crystalline vit B_{12} (Merck).

given Rose Bengal intravenously in physiological saline solution, in an amount of 100 mg/kg body weight.

The ultraviolet light exposure of the rabbits was the same as previously described(15).

The concentration of total urinary coproporphyrin (UCP) was determined daily by the method of Schwartz and co-workers(16). It should be emphasized that this method relates in the main to preformed porphyrin, and the values are therefore smaller than those obtained with a new and more sensitive procedure which includes whatever porphyrin is excreted as chromogen(17,18).

In each experiment the folic acid and the vit. B_{12}‡ were injected 10 minutes before the Rose Bengal. In those rabbits receiving 200-300 mg nicotinamide, the dosage was divided in 3 equal parts and given the same day on which the Rose Bengal was given. The first dose of nicotinamide was given between 8 and 9 A.M., the Rose Bengal between 10 and 11 A.M. and the second and third doses of nicotinamide between 12 and 2 P.M., and 4 and 6 P.M. respectively. The other rabbits received the drug 10 minutes before the Rose Bengal.

Results. The results are summarized in Table I in terms of the control value (Day 0) and the values for the first 3 days following treatment. Mean values are listed where more than one rabbit was employed at a given dose level. Though analyses were done for 6 days, the data for days 4-6 are omitted from the table since values for all groups returned to the normal range in this period.

In 5 control animals injected with Rose Bengal and exposed to ultraviolet light the range of peak values during the subsequent 24 hours was from 110 to 300 μg/24 hr. (These rabbits have been discussed in detail elsewhere) (15). This may be compared with a normal range in untreated rabbits of 3.2 to 14.2 μg/24 hr (mean = 7.3 μg/24 hr) as determined in a series of 50 rabbits.

Folic acid and B_{12} were very effective in limiting or preventing the usual coproporphyrinuria. With a dose of 45 mg of folic acid no increase in urinary coproporphyrin oc-

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16. Schwartz, S., Hawkinson, V., Cohen, S., and Watson, C. J., *J. Biol. Chem.*, 1947, v168, 133.

17. Schwartz, S., Zieve, L., and Watson, C. J., *J. Lab. and Clin. Med.*, 1951, v37, 843.

18. Watson, C. J., Pimenta de Mello, R., Schwartz, S., Hawkinson, V., and Bossenmaier, I., *J. Lab. and Clin. Med.*, 1951, v37, 831.

‡ Rubramin solution was provided through the courtesy of E. R. Squibb and Co. In certain experiments, as noted in Table I, crystalline B_{12} was used. This was provided through the courtesy of Merck and Co.

curred after Rose Bengal and ultraviolet light. The 5 and 15 mg doses, however, did not completely inhibit the effect. In general, vit. B_{12} in a dose from 5 to 35 μg almost completely prevented the increase of urinary coproporphyrin, while the combination of folic acid and B_{12} exhibited no special advantage.

Nicotinamide has a significant effect in reducing the coproporphyrinuria (type III) caused by the photodynamic action of ultraviolet light. Nevertheless, it is evident that the increases are only partially prevented.

Summary and Conclusions. The administration of large amounts of folic acid inhibited the increase of the urinary coproporphyrin in rabbits given Rose Bengal and exposed to ultraviolet light. The administration of small amounts of vit. B_{12} had the same effect. The combination of folic acid and vit. B_{12} had no advantage over either substance given separately. The administration of nicotinamide in large amount produced a significant but only partial inhibition.

In view of the fact that nicotinamide, folic acid, and vit. B_{12} are all intimately related to cellular metabolism, it seems highly likely that the marked increases of urinary coproporphyrin under these experimental conditions are likewise so related, but whether to the metabolism of the bone marrow, or some other system of cells is not clear. Further studies of these relationships are in progress.

I am deeply indebted to Dr. C. J. Watson and Dr. Samuel Schwartz for many valuable suggestions and interest in this work.

Received September 25, 1950. P.S.E.B.M., 1951, v77.

Effect of Total Body X-Irradiation on Glutathione Levels in Rats.* (18914)

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Recently the theory has been advanced that a relationship exists between x-irradiation effects and sulfhydryl groups. Barron(1) reported that irradiation decreases the metabolism of substances requiring sulfhydryl enzymes and other investigators that changes in glutathione levels affect the sensitivity of animals to irradiation,(2,3). Therefore, it appeared desirable to investigate glutathione levels following irradiation.

Method. Male and female Sprague-Dawley rats weighing from 170 to 275 g were used. Reduced glutathione in blood and liver was determined(4). Total glutathione was measured by treating the filtrates with H_2S at pH 8, acidifying, removing H_2S with CO_2, and determining total reduced glutathione. The difference between the glutathione before and after H_2S reduction was considered to represent oxidized glutathione. Known solutions gave good recoveries. Hematocrit values were obtained using 0.01 cc of blood(5). Somogyi's method was used to determine blood glucose(6). Reduced glutathione values on 0.2 cc of whole blood were determined one week before irradiation. Analyses one week apart showed no significant variation. No glutathione was found in rat plasma before or after irradiation. Red blood cell glutathione concentrations were calculated by dividing the whole blood glutathione value by the hematocrit and multiplying by 100.

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* Presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy by Ruth D. Peterson, June 1950. Supported by grants from the Diabetic Research Foundation of Portland, Ore., and the Rinehart Foundation of Wheeler, Ore.

1. Barron, E. S. G., *Nuclear Science Abst.*, 1948, v1, 369.

2. Patt, H. M., Tyree, E. B., Straube, R. L., and Smith, D. E., *Science*, 1949, v110, 213; Patt, H. M., Smith, D. E., Tyree, E. B., and Straube, R. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 18.

3. Cronkite, E. P., and Chapman, W. H., *Fed. Proc.*, 1950, v9, 329.

4. Bruckman, G., and Wertheimer, E., *J. Biol. Chem.*, 1947, v168, 241.

5. Van Allen, C. M., *J. Exp. Med.*, 1925, v45, 69.

6. Somogyi, M., *J. Biol. Chem.*, 1945, v160, 61, 69.

Duplicate determinations were done on 160 mg or less of liver (light nembutal anesthesia). No significant change was found when control biopsies were obtained at 2 week intervals. Eight series of 10 rats each were irradiated. Initial liver biopsies were taken in series 4, 5, and 6. Two weeks after biopsy initial whole blood glutathione determinations were made, and the rats were irradiated a week later. Only one biopsy was taken following irradiation; no blood glutathione values were determined after biopsy. The animals were subjected to 500 r total body irradiation in a round plywood box holding 10 rats in separate V-shaped compartments. Radiation factors were 200 KV., 15 ma., 0.5 mm Cu. + 1 mm Al. filters, 80 cm target distance, 1.28 mm Cu. half value layer, 24 r per minute dose rate.

Results and Discussion. The average initial reduced glutathione value in the blood of 88 control rats was 44 ± 5.0† mg %. The average initial calculated red blood cell glutathione was 104 ± 11 mg %. In 20 rats no significant deviation from initial values in blood glutathione levels were found 10 minutes to 24 hours post-irradiation. Thirteen rats picked at random 48 to 72 hours after x-irradiation showed a rise in blood glutathione values (t = 3.5). The average hematocrit value in these animals after irradiation was identical with that of the control animals. The calculated red blood cell glutathione value rose as high as 177 mg %. Since a relationship between adrenal glands and glutathione metabolism has been indicated(3,7,8) it is possible that this increase in glutathione is due to adrenal cortical activity. Rats confined in the irradiation box for the same length of time as irradiated animals showed no change in glutathione levels (n = 18, t = 0.02) or hematocrit values (n = 17, t = 0.04).

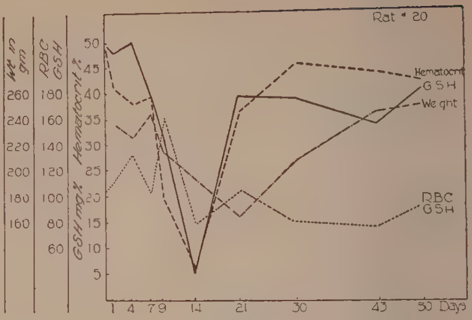

Figure I

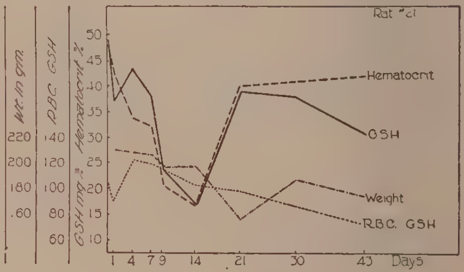

Figure II

FIG. 1 and 2. Showing the effect in rats of 500 r irradiation on hematocrit levels, blood glutathione values, calculated red blood cell glutathione values and weights.

Between 6 and 19 days post-irradiation, 37 of 45 rats showed reduced glutathione levels in blood more than twice the standard deviation below the control mean and 29 more than 3 times the standard deviation below the control mean. The blood glutathione values of 11 animals fell to 10 mg % or lower. However, even this low glutathione level was not necessarily fatal (Fig. 1). Our data are compatible with those of Schacter and Fishler(9).

Hematocrit values ordinarily decreased post-irradiation, at times irregularly in relation to blood glutathione. As blood glutathione fell, the RBC glutathione often rose above 142 mg % (more than 3 x the standard deviation above the control mean), but in some instances fell below 70 mg % (more than 3 x the standard deviation below the control mean). Some values continued to remain significantly low during recovery.

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† Standard deviation.

7. Edelman, A., *Fed. Proc.*, 1950, v9, 36.

8. Cronkite, E. P., Brechner, G., and Chapman, W. H., Naval Medical Research Institute, 1950, Project NM 006 012 05 02, Rep. No. III, Studies on the Mechanism of the Protective Action of Glutathione.

9. Shacter, B., and Fishler, M. C., U. S. Atomic Energy Commission Unclassified Document AD-214(B).

Fig. 1 and 2 are representative of 6 animals followed for over a month. Rat 21 was sacrificed on the 43rd day, at which time the liver glutathione concentration was significantly low (85 mg %).

Total glutathione levels were done on 3 rats with blood glutathione concentrations below 10 mg %. Only a small amount of oxidized glutathione was found, an average of 5 mg % for blood and 6 mg % for liver. Therefore, the low reduced glutathione values were not due to an increase in oxidized glutathione.

Initial values on 40 rats for reduced glutathione in liver averaged 184 ± 19 mg %. No change occurred before the 6th day following irradiation. Nine low liver glutathiones (47 to 116 mg %) were found in a total of 23 rats biopsied between 6 to 19 days post-irradiation. Reduced glutathione levels in the blood of these rats were 10 mg % or less.

Inanition was considered as a possible factor in this experiment(10). Forty-eight hours following irradiation a typical series of 10 animals (average weight 183 g) had lost 12 g. However, by the seventh day the average weight had returned to 185 g. Body weights then slowly decreased again. Nine rats with significantly low liver glutathiones (47 to 116 mg %) lost the same amount of weight as 7 rats with no change in liver glutathione values (162 to 222 mg %). At biopsy the stomach was palpated and food particles could be felt. When rats succumbed at biopsy food was always found in the stomach and small intestine. Six rats were starved until their average weight loss was 47 g; yet no decrease was found in liver reduced glutathione values (162 to 196 mg %). Paired feeding experiments were considered useless without determining intestinal absorption(11). However, 9 rats with blood glutathione concentrations from 9 to 20 mg % maintained their average blood glucose level at 120 mg %. Thirteen irradiated animals starved for 24 hours had an average blood glucose level of 76 mg %. Therefore, it was concluded that inanition does not explain the decrease in glutathione levels in liver.

Glutathione may function in the tissues to maintain enzymes in their active SH form. Therefore, it is reasonable to assume that low glutathione levels may interfere with metabolic processes.

Summary. A study has been made of blood and liver glutathione after 500 r total body x-irradiation. The data show first, that irradiation generally causes a decrease in blood glutathione; and second, in some cases, a decrease in liver glutathione.

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10. Binet, L., and Weller, G., *Compt. rend.*, 1935, v119, 939.

11. Bennett, L. R., Bennett, V. C., Shaver, A., and Grachus, T., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 439; Lauran, M., and Lartigue, O., *Compt. rend.*, 1950, v230, 1426; Buchwald, K. W., *J. Exp. Med.*, 1931, v53, 827.

Received March 19, 1951. P.S.E.B.M., 1951, v77.

Crystal Structures in Dried Smears of Gastric Mucus.* (18915)

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In the course of our studies on acid-free canine mucus, we have examined a large number of specimens microscopically. Air-dried smears were stained with toluidine blue, by a technic previously described(1)[†]; Wright's stain was also used occasionally.

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* This investigation was supported by a research grant from the National Cancer Institute, of the National Institutes of Health, United States Public Health Service.

1. Hess, M., and Hollander, F., *J. Lab. Clin. Med.*, 1944, v29, 321.

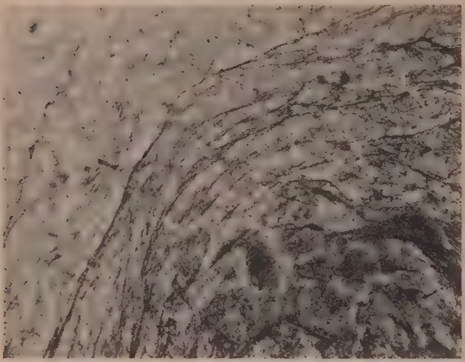

FIG. 1. Smear of transparent gastric mucus (canine) showing long strands of the secretion. Toluidine blue. 38×.

The specimens often contained numerous epithelial and other cells(2), interspersed among small cell-free areas, but many smears were entirely acellular. This was particularly true for "spontaneously" secreted mucus, collected from resting gastric pouches without the application of a mucigogue.‡ The present paper reports the varied microscopic appearance of such acellular mucus, as revealed by careful study of hundreds of specimens.

Observations. Smears free of whole cells often contain particles suggestive of cellular debris, with or without a reticulated substratum. Sometimes the smears reveal only dark wisps or long strands of mucus more viscous than the secretion in adjacent areas (Fig. 1). At other times they contain minute vacuoles which may be bubbles of air incorporated by shaking prior to smearing, or as has been reported elsewhere(3), possibly bubbles of CO_2 released spontaneously by the mucus. Frequently, however, the smears contain fern-like crystal structures of variable size (Fig. 2) and these sometimes occur as rectangular outgrowths from cubic nuclei (Fig. 3) or in stellate patterns about a central point of origin. Another type of structure observed in these smears resembles fronds of seaweed; these also may be isolated (Fig. 4) or may appear as symmetrical outgrowths from a central nucleus (Fig. 5). Examination of this nucleus at high power suggests that it is compounded of the same globular elements

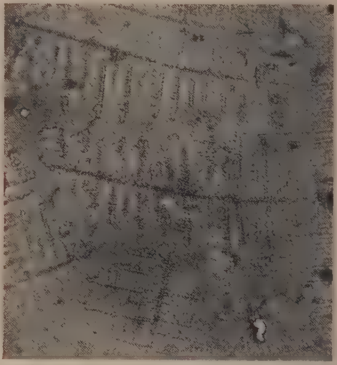

FIG. 2. Smear of transparent gastric mucus (canine), showing rectangular fern-like crystals. Toluidine blue. 38×.

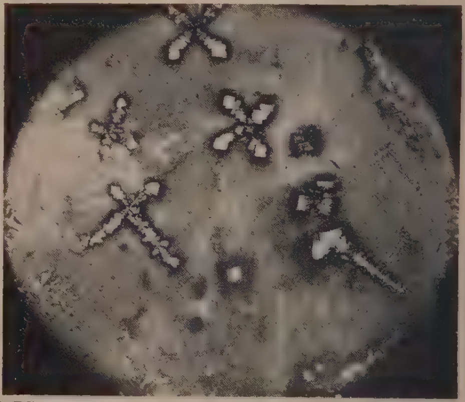

FIG. 3. Smear of transparent gastric mucus (canine), showing relatively large cubic (?) crystals with occasional fern-like structures growing out of them. Toluidine blue. 38×.

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† This staining technique was designed for the preparation of smears in which the metachromatic staining is permanent. Attention is therefore called to the fact that the metachromasia of the specimens discussed in this report is generally as pronounced at this time as when the smears were originally prepared, 8 or more years ago. For a modification of this technique applicable to tissue sections, see Hess and Hollander, *J. Lab. Clin. Med.*, 1947, v32, 905.

2. Hollander, F., Stein, J., and Lauber, F. U., *Gastroenterology*, 1946, v6, 576.

‡ A method for stimulating the secretion of pure, acellular mucus in relatively large quantities has recently been developed by Janowitz, Hollander, and Jackson, Proc. Soc. Exp. Biol. and Med., 1951, v76, 578.

3. Hollander, F. and Lauber, F. U., *Fed. Proc.*, 1948, v7, 56.

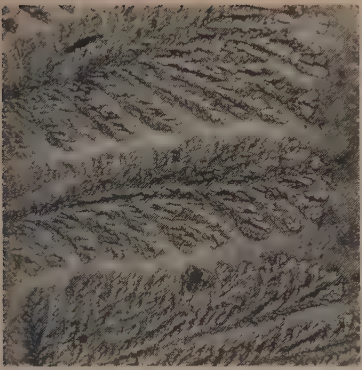

FIG. 4. Smear of transparent gastric mucus (canine), with seaweed-like structures. Toluidine blue. 38×.

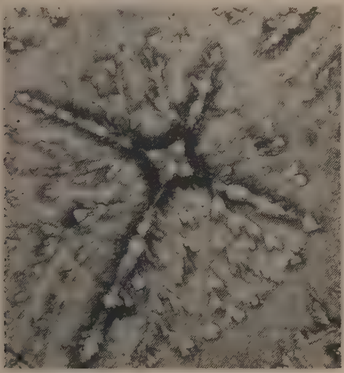

FIG. 5. Smear of transparent gastric mucus (canine), showing seaweed-like structures growing out symmetrically from a central nucleus. Toluidine blue. 38×.

as are visible in the outgrowths from its corners, rather than a cube as in Fig. 3.

These crystals have never been seen to take Wright's stain or toluidine blue. With the latter there is usually a concentration of metachromatic material at their margins, corresponding to the dark lines of variable thickness in some of the photomicrographs. Sometimes, careful examination reveals a thin metachromatic network over their surfaces as well, but the crystals themselves are unstained.

Comment. Failure to react with toluidine blue suggests that these crystals are not gastric mucins. Pepsinogen also is ruled out by reason of its possessing a different crystalline appearance, as reported by Northrop *et al.* (4). Pepsin could not be present because the mucus specimens had not been acidified before smearing. Some inorganic salt such as NaCl is likewise a possibility. Henning and Norpoth(5) offered such an explanation for the "lattice structures" (Gitterbildungen) observed microscopically in unstained dried drops of filtered human gastric juice. These structures, which included both simple cubic crystals and delicate fern-like structures, resembling the crystals reported herewith for relatively pure pouch mucus, were believed to be crystals of NaCl, modified by the presence of foreign substances from saliva or regurgitated duodenal fluid. However, these investigators offered no evidence to support their contention, nor have we been able as yet to demonstrate that the crystals seen in non-acid mucus are NaCl. On the contrary, it is now generally recognized that such rectangular crystal forms are non-specific(6), and a similar skeletal growth with successive branching has been grown from pure NH_4Cl by Bunn(7). It may be suspected that these crystals are artefacts, deriving from the stains used in preparing the specimens, but this can not be the case because we have watched the patterns in Fig. 2 and 5 growing under the microscope in a slowly drying *unstained* preparation. It is evident, therefore, that characterization of the mucus crystals as NaCl is not warranted without direct chemical evidence. Further attempts to identify these structures will be of interest, but they must be made with the aid of suitable microchemical methods.

Conclusion. Several characteristic crystal patterns have been observed microscopically in dry smears of pure gastric mucus from

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4. Northrop, J. H., Kunitz, M., and Herriott, R. M., Crystalline Enzymes, Columbia University Press, New York, 2nd Ed., 1948.

5. Henning, N., and Norpoth, L., *Z. f. klin. Med.*, 1933, v126, 1.

6. Chamot, E. M., and Clyde, W. M., Handbook of Chemical Microscopy, John Wiley & Sons, Inc., New York, 2nd Ed., 1938, v1, pp. 333 and 335.

7. Bunn, C. W., Chemical Crystallography, The Clarendon Press, Oxford, 1945. (Fig. 8, Plate I).

canine Heidenhain pouches. Although their chemical composition has not been established, there is reason to believe that they are neither pepsinogen, pepsin, nor one of the mucins.

Received Apr. 25, 1951. P.S.E.B.M., 1951, v77.

Effects of Cortisone and 11-Desoxycortisone on Pain Thresholds in Man.*

(18916)

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While testing 11-desoxycortisone† in allergic disorders, it was noted that the tooth pain thresholds of 5 patients decreased with this steroid medication. Thus the patients, over a period of hours, were able to recognize a smaller stimulus. Since these patients were not trained, the decreases in pain thresholds could be, and tentatively were, interpreted as a normal learning curve. Because cortisone and also ACTH therapy occasionally produce euphoria and other psychic changes(1,2), it is remotely possible that the mechanism of the analgesic effect of cortisone is similar to that of the opiates.

Method. To test these hypotheses, trained subjects were used to assay the effects on pain thresholds of 11-desoxycortisone and cortisone. As a control for this study a placebo (cholesterol) was administered. Absolute blind test conditions were used in that the vials of suspended steroid were labeled A, B, and C and the identity of the injected suspension was unknown both to the experimenter and the subjects.

Four trained male subjects were used for these tests. At the beginning of each experiment 2 or more control pain thresholds were determined with both the tooth and warm wire algesimeters. The saline suspension of steroid (25 mg) was administered by intramuscular injection and the pain thresholds were then measured at hourly intervals for 6 hours. The experimental period extended over 7 hours and the subjects were permitted to resume their usual activities between pain threshold determinations. Each of the 4 subjects received 11-desoxycortisone, cortisone, and the placebo (cholesterol) at weekly intervals in an arbitrary but varied sequence. Two types of algesimeters were used: the warm wire algesimeter of Lee, Williams, and Pfeiffer(3), and the tooth algesimeter of Goetzl, Burrill, and Ivy(4). The warm wire algesimeter consists of a nichrome resistance wire (crimped to provide a tip for application of the stimulus) in series with a milliammeter, a rheostat, and a source of electrical current. The current was varied by means of the rheostat. The instrument was calibrated in increments of 10 milliamperes with a range of 400-1000 milliamperes. The conduction heat stimulus was applied to the skin of the volar surface of the wrist. The tooth algesimeter provided induced alternating current as a secondary coil was moved toward a rotating primary coil. The calibration was in increments of 0.2 volt with a range of 0 to 4.2 volts The induced electrical stimulus was applied to the filling of a tooth. Two pain thresholds were determined with the algesimeters. Using the warm wire algesimeter on the skin of the volar surface of the wrist, the wrist pain threshold was

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* This investigation was supported in part by a research grant from the National Heart Institute, of the National Institutes of Health, Public Health Service.

† Compound S of Reichstein.

1. Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F., *Arch. Int. Med.*, 1950, v85, 545.

2. Boland, E. W. and Headley, N. E., *J. A. M. A.*, 1949, v141, 301.

3. Lee, R. E., Williams, H. L., and Pfeiffer, C. C., *Fed. Proc.*, 1949, v8, 314.

4. Goetzl, F. R., Burrill, D. Y., and Ivy, A. C., *Quart. Bull. Northwestern Univ. Med. School*, 1943, v17, 280.

determined. The wrist was immersed in a 40°C water bath for one minute just prior to testing. The wrist threshold was taken as the milliamperage which consistently produced 3 painful stimuli with 3 applications of the wire. The stimulus was applied to the same spots unless they became erythematous or altered in sensitivity as compared with adjacent areas. The subject was made aware of the sensation of touch with a cool wire stimulator to prevent confusion of touch with slight pain. With the tooth algesimeter 2 endpoints are frequently used. The first is the perception of a non-painful sensation and the second is the perception of true pain. In these experiments the endpoint was the perception of true pain. For a description of the method of determining the tooth pain threshold see the report of Sonnenschein, Jenney, and Pfeiffer(5).

The data were subjected to statistical analysis applying Student's "t" as described by Snedecor(6). The pain threshold alteration produced by 11-desoxycortisone or cortisone was compared in each individual with the alteration produced by the placebo (cholesterol) in the same individual. A test of significance was made of the mean of these differences. The usual confidence level of 5% was employed.

Results. 11-desoxycortisone did not significantly alter the wrist pain threshold, but it did lower the tooth pain threshold significantly. The tooth threshold decreased gradually to the lowest reading 5 hours after the injection. These results are summarized in Table I. Cortisone did not significantly alter the wrist or tooth pain thresholds (Table I). The threshold alteration produced by 11-desoxycortisone was compared in each individual with the alteration produced by cortisone in the same individual. The significance of the mean of these differences was determined. The differences between the alterations of the wrist and tooth pain thresholds produced by 11-desoxycortisone and cortisone were not significant (Table I).

TABLE I.

	Hr after inj					
	1	2	3	4	5	6
Effects of 11-desoxycortisone on pain thresholds (25 mg in 4 subjects)						
Pain threshold	*	*	*	*	*	*
Tooth (volts)	−.25	−.28	−.33	−.35	−.38†	−.13
Wrist (m.a.)	5	−12.5	−2.5	2.5	17.5	5
Effects of cortisone on pain thresholds (25 mg in 4 subjects)						
Pain threshold	*	*	*	*	*	*
Tooth (volts)	−.18	−.15	−.10	−.05	.08	−.05
Wrist (m.a.)	0	−17.5	5	0	22.5	35
Comparison of effects of 11-desoxycortisone (25 mg) and cortisone (25 mg) on pain thresholds (4 subjects)						
Pain threshold	†	†	†	†	†	†
Tooth (volts)	.08	.13	.23	.15	.45	.08
Wrist (m.a.)	−5	−5	7.5	−2.5	5	30

* Mean of individual differences between threshold alterations produced by the drug and the placebo in the same individuals.

† Mean of individual differences between threshold alterations produced by 11-desoxycortisone and cortisone in the same individuals.

‡ Statistically significant, *i.e.*, probability for random selection calculated from "t" values for the mean differences is less than 5%. ($P = < .05$).

(m.a.) = milliamperes.

The side effects reported to the operator after the injection of these agents were as follows: After cortisone, Subjects 1 and 2 experienced headache, and Subjects 2 and 3 experienced euphoria; after 11-desoxycortisone, Subject 2 experienced marked sleepiness and xerostomia; and after cholesterol, no symptoms were reported.

Discussion. The lowering of the tooth pain threshold produced by 11-desoxycortisone confirms the previous findings in allergic patients. In the present study, trained subjects were used and a placebo injection was employed as a control under blind-test conditions. This lowering of the tooth threshold can now be interpreted as the effect of the drug rather than the effect of a normal learning curve. However, the wrist pain threshold was not lowered by 11-desoxycortisone. The

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5. Sonnenschein, R. R., Jenney, E. H., and Pfeiffer, C. C., *J. Appl. Physiol.*, 1949, v2, 141.

6. Snedecor, G. W., *Statistical Methods*, Iowa State College Press, Ames, 1946, p. 43.

only other compound which is known to lower the pain threshold is ergotamine tartrate. This agent lowers the tooth pain threshold and also the warm wire pain threshold.

Cortisone failed to raise the pain thresholds although euphoria was experienced by 2 of the 4 subjects. These results do not support the hypothesis that cortisone produces analgesia by the same mechanism as the opiates. Cortisone and the antipyretic analgesics have many similar actions. Similar metabolic and biochemical effects of cortisone and antipyretic analgesics are(7): 1. antipyretic effect, 2. glycosuria, 3 uricosuric effect, and 4. dilution of blood. On the glycosuria of the depancreatized rat, salicylates have an effect opposite to that of cortisone(8). Both cortisone and the antipyretic analgesics produce pain-relief only in certain selected clinical disorders. Sonnenschein and Ivy(9) found that the elevations of the tooth pain threshold after aspirin were not significant when compared with a lactose placebo. Studies in our laboratory confirmed this failure of aspirin to elevate the tooth threshold and further demonstrated the failure of aspirin to elevate the warm wire pain threshold. However, both the tooth and warm wire methods of algesimetry have demonstrated statistically significant pain threshold elevations in trained subjects with morphine and other analgesics. As cited above, cortisone and the salicylates are similar in many respects and this study indicates one further similarity in that neither produces an increase in pain thresholds.

The potent analgesics act on the physiologic system concerned with pain (the receptors, nerve fibers, synapses, or higher mental centers). However, pain-relief can be effected by agents that remove the pathophysiologic state causing the stimulation of pain receptors. These findings suggest that cortisone, like the salicylates, may produce pain relief by altering pathophysiologic states. That cortisone may alter pathophysiologic states is supported by numerous observations. With cortisone therapy there has been reported: a decrease in the joint swelling of rheumatoid arthritis and acute rheumatic fever; suppression of fever, skin eruption, and serositis in acute disseminated lupus erythematosus; subsidence of ocular inflammation; and a decreased edema in nephritis(10). Further speculation as to the mechanism of clinical pain relief by cortisone must await additional experimental investigation.

Summary. Under the conditions of these experiments, 11-desoxycortisone (25 mg) lowered the tooth pain threshold, but did not alter the wrist pain threshold, and cortisone (25 mg) did not alter the tooth or wrist pain thresholds. An alteration of the pathophysiologic state initiating pain is suggested as an explanation for the pain relief afforded by cortisone.

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7. Pfeiffer, C. C., Lee, R. E., Hemmens, E. S., and Hasegawa, A., *Proc. Inst. of Med. of Chicago*, 1950, v18, 96.

8. Ingle, D. J., Proc. Soc. Exp. Biol. and Med., 1950, v75, 673.

9. Sonnenschein, R. R., and Ivy, A. C., *J. Pharm. and Exp. Therap.*, 1949, v93, 308.

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Received May 2, 1951. P.S.E.B.M., 1951, v77.

Aqueous Humor as a Tissue Culture Nutrient.* (18917)

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Since Harrison(1) first performed experiments on the cultivation of cells *in vitro* using frog lymph, a variety of body fluids has been used in nutrient media. In view of the results of Greene(2) and Browning(3) on the transplantation of tumors and embryonic tissues to the anterior chamber of rodents' eyes, a study was undertaken to evaluate aqueous humor as a tissue culture nutrient. The present report deals with results obtained with this medium in the cultivation of fibroblasts. The necessity of using a plasma clot has been avoided by the use of perforated cellophane in the case of fibroblasts, but the difficulty encountered in growing epithelial cells in the absence of a solid organic substrate has rendered results with these cells inconclusive as yet.

Materials and methods. The small size of rodents' eyes precluded their use as a source of aqueous humor. An inexpensive source of fairly large amounts of fluid was available in the eyes of adult cattle killed at a nearby slaughter house. Eyes were removed by attendants at the slaughter house without sterile precautions. In the laboratory the corneal surfaces of the eyes were bathed with 70% alcohol and the fluid was removed by means of a sterile 20 gauge hypodermic needle and syringe. Each eye yielded 1.5-2.0 cc of crystal clear fluid which did not clot on standing. Eyes in which either chamber had been damaged in removal or eyes which showed gross pathology were discarded. It was found convenient to store the fluid in the refrigerator without preservative in 10 or 20 ml aliquots. Bacterial contamination was rare when these simple technics were used. Several series of experiments in which aqueous humor was used were performed over a period of 1½ years, using both roller tubes and Carrel flasks. In the experiments to be reported, Carrel D 3.5 mm flasks were used. The cellophane was prepared after the manner of Evans and Earle(4) except that it was more convenient to insert the discs when wet and to sterilize the cellophane-containing flasks. The hearts from 12-day-old chicks were used as the explant material. These were cut into 4 pieces, each measuring about 2 mm square. One such fragment was placed in the approximate center of each flask, under the cellophane, after the addition of nutrient. The flasks were divided into 3 groups: (1) those containing aqueous humor only; (2) those containing embryo extract§ 20%, aqueous humor 80%; (3) those containing embryo extract§ 20%, horse serum‖ 40%, Earle's saline 40%. A total of 1.5 cc of nutrient was added to each flask just before the tissue fragment was put in place. The nutrients were changed twice a week by removing the fluid with suction, rinsing each flask with 1 cc of Earle's saline, and adding 1 cc of fresh nutrient. The cultures were incubated at 38.5°C without shaking or agitation. The flasks were not gassed, but phenol red indicator in the nutrient demonstrated that the pH remained close to 7.5.

Results. The fragments bathed in 100% aqueous humor showed migration of cells for the first 3 days. After this initial period further migration was not apparent nor was there unequivocal evidence of actual cell proliferation. The cells were clear in outline and contained very few droplets or unusual granulations. The cultures thereafter remained essentially static except for slow distintegration of the marginal cells. Despite the lack of activity, the cells of the explants survived at least 6 weeks as indicated by fixed preparations (Fig. 1).

In the group containing 20% embryo extract and 80% aqueous humor, cell migration and proliferation occurred early and persisted. The cell outline and clarity of cytoplasm re-

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* This investigation was done in part under fellowships from the American Cancer Soc., Inc., recommended by the Committee on Growth of the National Research Council, the Jane Coffin Childs Memorial Fund for Medical Research, and the Rotary Foundation of Rotary International.

† Fellow of the American Cancer Society, Inc.

‡ Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

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2. Greene, H. S. N., *Exp. Med.*, 1941, v73, 461.

3. Browning, H. C., *Cancer*, 1949, v2, 646.

4. Evans, V. J., and Earle, W. R., *J. Nat. Cancer Inst.*, 1947, v8, 103.

§ Difco TC desiccated embryo extract; reconstituted with double distilled water and diluted 1:5 with Earle's saline. A cloudy suspension was formed which after centrifugation at 1500 RPM yielded a slightly cloudy supernatant.

‖ Difco TC desiccated horse serum; reconstituted with double distilled water. This resulted in a clear solution.

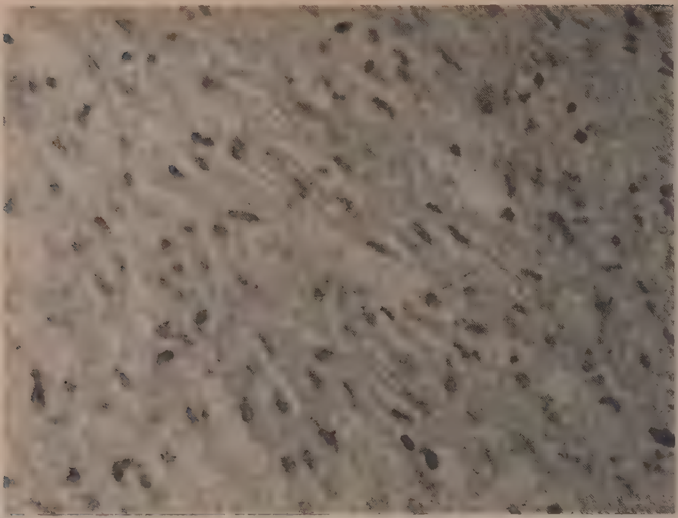

FIG. 1. Paraffin section through explant maintained in 100% aqueous humor for 6 weeks. Beginning necrosis is indicated by occasional pyknotic nuclei. H and E. × 300.

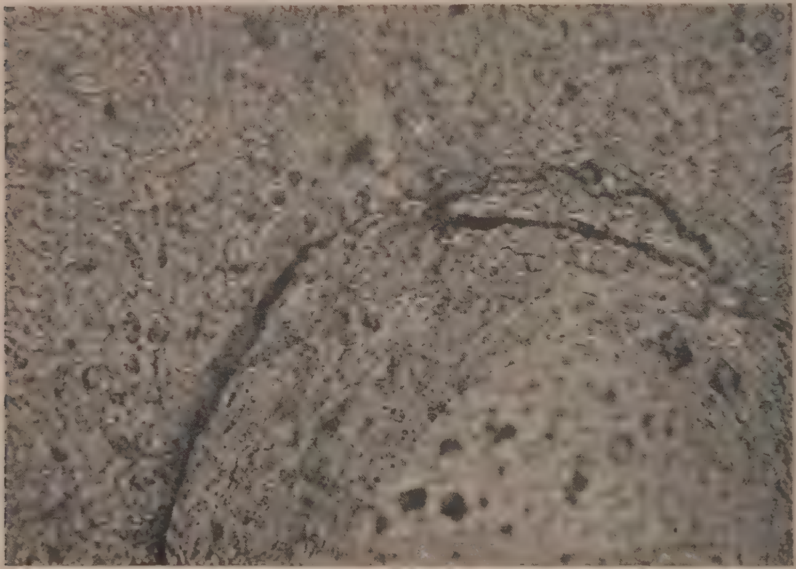

FIG. 2. Living culture under perforated cellophane in 80% aqueous humor and 20% embryo extract, photographed at 6 weeks. Cells are forming a characteristic whorl inside the perforation. Growth outside the perforation is several cells in thickness. × 175.

sembled that of cells grown in 100% aqueous humor. Growth occurred on both the glass and cellophane surfaces and tended to be in the form of continuous sheets, several cells in thickness (Fig. 2). Transplantation was performed at six weeks although the cells covered only about one-half of the flask area. Just prior to transplantation, the marginal cells had become somewhat granulated and proliferation appeared to be reduced. After transplantation, cells again migrated and proliferated from the transplanted fragment (Fig. 3).

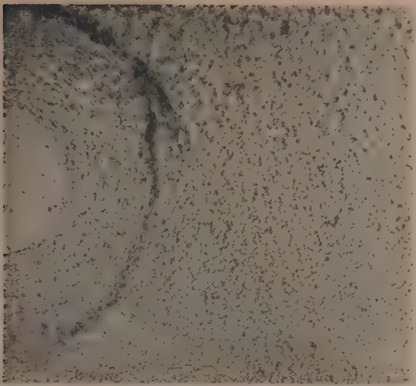

FIG. 3. Living culture under perforated cellophane in 80% aqueous humor and 20% embryo extract, photographed at 14 weeks, 8 weeks after transplantation. Luxuriant and uniform growth is evident. × 80.

In flasks which were continued without transplantation, cells were viable and active at 14 weeks although a zone of necrosis had appeared at the margins and was slowly increasing in width. Further observations over a longer period were not possible because of fungus contamination of the entire series.

Growth of fibroblasts from fragments placed in 20% desiccated embryo extract, 40% desiccated horse serum and 40% Earle's saline varied considerably from flask to flask, in contrast to the very consistent results obtained in both aqueous humor groups. In general, the cells in the former medium migrated slowly, tended to separate from the explant margin and to proliferate in the form of clumps of cells which later coalesced. The average area of growth produced at the time of transplantation was approximately one-half that produced in the group grown in aqueous humor and embryo extract, although growth in the best flask surpassed that of the aqueous humor group. On transplantation from this flask the superiority did not persist although migration and proliferation from the new transplants occurred earlier than from the aqueous humor transplants. The individual cells contained larger and more numerous vacuoles and granules and there was more variation in cell type than with the aqueous humor group. These observations have been confirmed in similar studies using roller tubes.

Discussion. The composition of aqueous humor has been the subject of considerable investigation(5). The ionic differences between it and serum are rather small and within the limits tolerated by living cells. The most striking differences are low protein (and low antibody) content and high ascorbate values found in aqueous humor.

The concentration of protein approaches the order of magnitude found in interstitial fluid. The importance of this factor is uncertain, but the correspondingly low antibody level may reduce inhibitory influences. The much higher levels of protein in serum may be the explanation for the toxic effects of some sera. The concentration of ascorbate in aqueous humor is about 12 times that in plasma. The specific vit. C levels required in fibroblast cultures are unknown, but large amounts of ascorbate are found in both embryonic and tumor tissue(6). This may possibly reflect higher needs of vit. C for optimal growth than are provided for in desiccated horse serum. In this laboratory the use of aqueous humor and desiccated embryo extract gave results superior to the desiccated horse serum embryo extract and saline mixture in respect to rapidity, consistency and extent of growth, uniformity of cell morphology and clarity of cytoplasm. It should be indicated that the results obtained using serum, saline and embryo extract reported in this series fall short of the extensive growth reported by Evans and Earle(4) using freshly prepared media. Possible explanations for this discrepancy may be the use of 12-day instead of 10-day embryos and 2 instead of 3 changes of fluid per week. Both of these factors were, however, applicable to all groups of the series. A further factor to be considered is the use of desiccated horse serum. Difco TC desiccated serum was used in the belief that since it had been tested and found satisfactory by the Tissue Culture Commission, it would furnish the most dependable control medium for comparison with aqueous humor. Horse serum

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5. Kinsey, V. E., *Arch. Ophthal.*, 1950, v44, 215.

6. Bicknell, F., and Prescott, F., The Vitamins in Medicine, New York, Grune and Stratton, 2nd ed., 1946.

is, however, known to vary considerably and, moreover, the drying procedure may possibly result in an inferior serum from the standpoint of volume of growth. On the other hand, Hetherington has reported excellent results with desiccated media(7,8).

The efficacy of aqueous humor as a tissue culture nutrient suggests that the success of intra-ocular transplantation depends on the ability of the fluid to maintain cells for relatively long periods or until vascularization occurs permitting continued growth.

Summary. (1) Pure aqueous humor from beef eyes will support survival but only limited growth of chick fibroblasts. (2) A mixture of aqueous humor and 20% desiccated embryo extract results in extensive proliferation of chick fibroblasts which is superior to that obtained in media prepared from desiccated horse serum, embryo extract and saline. (3) The possible explanations and significance of these findings have been discussed.

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Received May 2, 1951. P.S.E.B.M., 1951, v77.

Antithyrotoxic Effects of Antibiotics in Rats.* (18918)

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Several antibiotics have recently been shown to exert pronounced growth effects when fed in small quantities to chicks(1,2), pigs(3,4), or rats(5). These effects can be obtained on diets either adequate or deficient in vit. B_{12}(2,6), indicating that several factors may be made available through the action of the antibiotics. Ershoff(7,8) recently reported that supplements of dried penicillin mycelia or aureomycin mash, but not crystalline aureomycin HCl, prolonged the average survival rate of immature rats fed massive doses of thyroid powder. Since vit. B_{12} has been shown to be effective in counteracting the growth-retardation induced in young rats by administering large amounts of thyroid-active substances(9-11), it was of interest to see whether crystalline penicillin, neomycin or streptomycin would be similarly effective on rations either adequate or deficient in vit. B_{12}.

Procedure. In the first experiment, 50 young female albino rats (Carworth) were fed the following basal ration for 36 days: yellow corn meal, 35%; ground wheat, 25%; whole milk powder (Borden), 20%; linseed oil meal, 10%; alfalfa leaf meal, 6%; brewers yeast, 3%; and table salt, 1%. This ration is believed to contain an adequate supply of vit. B_{12},[†] and only one shipment of whole milk powder during the past 2 years was found to

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* Published with the approval of the Director of the Michigan Agric. Exp. Station as Journal Article No. 1250.

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TABLE I. Effects of Protamone and Antibiotics on Body Growth, Food Intake and Oxygen Consumption of Young Female Rats on Vitamin B_{12}-Adequate Diet.

Treatment	No. of rats		Avg body wt, g			Avg food intake, g			Avg O_2 consump/hr/100 g body wt, ml
	Orig	Final	Orig	Final	Gain	Total	Daily[*]	Per g gain in body wt	
1. Controls	10	10	145[†] (135-151)	181[†] (168-190)	36.5	393.8	10.9	10.8	179.5 ± 6.5[‡]
2. Protamone	10	9	133 (128-147)	155 (114-174)	22	484.1	13.5	22	312.8 ± 32.9
3. Protamone + streptomycin	10	9	139 (130-148)	164 (118-170)	24.3	492.5	13.7	20.2	280.4 ± 30.7
4. Protamone + penicillin	10	10	144 (129-150)	185 (156-195)	41	548.6	15.2	13.3	284.7 ± 19.8
5. Protamone + neomycin	10	9	137 (128-148)	188 (160-194)	50.5	550.8	15.3	10.9	272.5 ± 21.2

[*] Corrected for deaths.

[†] Weights expressed to nearest whole number.

[‡] Standard error of mean.

be low in vit. B_{12}(12,13). This ration was supplemented with 0.16% Protamone[‡] and either procaine penicillin G, streptomycin sulfate or neomycin sulfate[§] in amounts of 0.05%. Body weight and food intake data were collected on alternate days. At the end of the experiment, basal oxygen consumptions were determined for each rat in a closed circuit Regnault-Reiset apparatus of the type described by Brody (14) and modified by Dr. E. P. Reineke of this department. The rats were fasted 15 hours prior to each test, and oxygen consumption was measured for 1½ hours. This procedure was repeated after 48 hours and the two values obtained for each rat were averaged. In all cases, there was less than 5% difference between the two figures obtained for each rat.[‖] In the second experiment, 50 young male albino rats (Dixboro) were fed for 42 days the same ration as used in the previous experiment, except that soybean oil meal[¶] was substituted for the whole milk powder. This ration, therefore, was free of animal protein and presumably of vit. B_{12}.[**] On the 42nd day, the rats were killed and the adrenals, spleen and thymus were removed and weighed. All rats were housed in wire bottom cages, in an air conditioned room at a temperature of 75 ± 1°F.

Results. The data from the first experiment are given in Table I and Fig. 1. The control rats (Group 1), which received neither Protamone nor antibiotic, gained an average of 36.5 g each and consumed an average of 10.9 g of food daily. The rats which received Protamone alone (Group 2), gained only an average of 22 g despite the greater average daily food intake. The efficiency of body-weight gains was reduced to about half, since about twice as many g of food were consumed

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[†] Numerous generations of rats have been reared on this ration during the past 15 years at Michigan State College.

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[‡] Protamone is the commercial name of an iodinated casein product containing thyroxine, and was kindly supplied by Cerophyl Labs., Kansas City, Mo.

[§] The authors wish to express their appreciation to Dr. D. F. Green of Merck and Co., Rahway, N. J., for the procaine penicillin G, and to Dr. Gordon Stocking of The Upjohn Co., Kalamazoo, Mich., for the neomycin sulfate and streptomycin sulfate.

14. Brody, S., Bioenergetics, 1945, Reinhold Publishing Corp., New York, N. Y.

[‖] The authors wish to express their appreciation to Miss Y. S. L. Feng for her careful work in determining the oxygen consumption of these rats.

[¶] Low fiber, solvent extracted, containing 50% protein. Manufactured by Archer-Daniels-Midland Co., Minneapolis, Minn.

[**] The addition of vit. B_{12} to this ration considerably enhances the growth rate of both strains of rats used in this study.

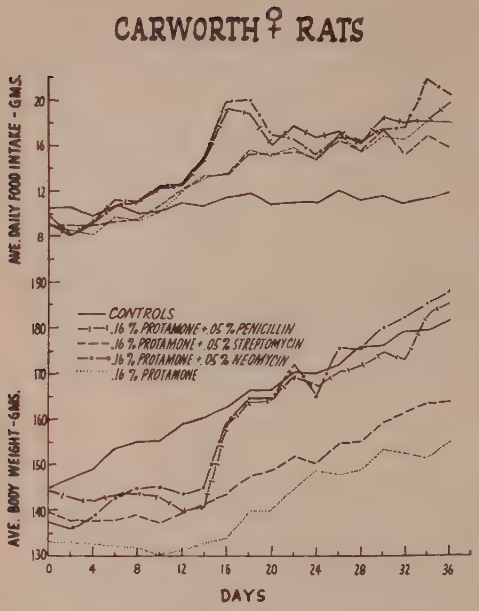

FIG. 1. Effects of Protamone and antibiotics on body growth and food consumption of female rats.

for each g of increment in body weight. The average basal oxygen consumption of these rats was increased 74% above the controls, indicating the presence of a severe degree of hyperthyroidism.

The addition of streptomycin sulfate (Group 3) apparently did not alter the results obtained with Protamone alone. However, procaine penicillin G (Group 4) or neomycin sulfate (Group 5) completely counteracted the Protamone-induced retardation of growth. The increase in food intake and body weight occurred rather abruptly, beginning on or about the 14th day of the experiment. The efficiency of body-weight gains was increased considerably above the Protamone-treated rats. Neither antibiotic significantly counteracted the increase in basal oxygen consumption induced by the Protamone.

The results of the second experiment (Table II), in which the animals were fed a diet presumably deficient in vit. B_{12}, were essentially similar to those obtained in the previous experiment. However, there were 3 notable exceptions. First, the antibiotics were relatively less effective in overcoming the inhibition of growth induced by the Protamone. Not only did the antibiotics fail to completely counteract the growth retardation, but it took 26 to 28 days before they showed any favorable effects on growth at all. The fact that the ration was deficient in vit. B_{12} may account to some degree for this reduced effectiveness of the antibiotics. Secondly, streptomycin sulfate was at least partially effective in increasing body weight, in contrast to its complete ineffectiveness in the first experiment. Finally, the mortality rate was increased by the antibiotics. All deaths occurred before the antibiotics exerted any favorable effects on growth.

The following data were obtained on the organ weights of the male rats. The adrenals of the controls averaged 14.1 ± 2.2 mg per 100 g of body weight, while those of the Protamone-treated rats averaged 38 ± 7.5 mg. This increase in adrenal weight is typical of hyperthyroid rats. Spleen weight was not altered either by Protamone alone or when combined with any of the antibiotics. Thymus weight in the controls averaged 160.1 ± 16.2 mg per 100 g of body weight and 107.1 ± 17.7 mg in the Protamone-treated rats, indicating an increased release of steroids from the adrenal cortex. Streptomycin and penicillin partially and neomycin completely counteracted the Protamone-induced reduction in thymus weight.

Discussion. These data indicate that crystalline penicillin, neomycin or streptomycin can partially or completely counteract growth retardation in young rats given large doses of Protamone. At least 2 of the antibiotics, penicillin and neomycin, appeared to be more effective in the presence of adequate amounts of vit. B_{12}, as suggested in the first experiment. Although all 3 antibiotics were at least partially effective on the ration deficient in vit. B_{12}, as seen in the second experiment, the possibility cannot be ruled out that these rats obtained some animal protein factor from the fecal material seen adhering to their tails or on the food containers. There may also have been a sex difference in the response of the rats to the antibiotics.

It is interesting that normal growth can

TABLE II. Effects of Protamone and Antibiotics on Body Growth and Food Intake of Young Male Rats on Vitamin B_{12}-Deficient Diet.

Treatment	No. of rats		Avg body wt, g			Avg food intake, g		
	Orig	Final	Orig	Final	Gain	Total	Daily*	Per g gain in body wt, g
1. Controls	10	9	130† (124-135)	246† (217-281)	115.9	612	15	5.3
2. Protamone	10	8	130 (126-136)	161 (126-196)	31.8	823.6	22.3	26.1
3. Protamone + streptomycin	10	6	129 (125-134)	182 (136-233)	53.4	861.4	26.9	16.1
4. Protamone + penicillin	10	6	129 (124-134)	196 (163-207)	66.8	887.8	25.9	13.3
5. Protamone + neomycin	10	7	129 (124-135)	178 (157-232)	49.2	839.6	23.1	17

* Corrected for deaths.

† Weights expressed to nearest whole number.

proceed in severely hyperthyroid rats in the presence of adequate amounts of vit. B_{12} and/or antibiotics. The great increase in basal metabolism effected by the Protamone, as indicated by the 74% increase in oxygen consumption, was not reduced significantly by the antibiotics. However, the antibiotics, like vit. B_{12}(15), increased both daily food consumption and ability to convert food into body-weight gains. The "thyrotoxicosis" of severe hyperthyroidism may largely be the manifestations of a multiple vitamin deficiency, as previously suggested(7).

The failure of the antibiotics to alter the increase in adrenal weight induced by the Protamone can be considered as evidence that they do not alter the turnover of Protamone in the body. Similarly, vit. B_{12} is not believed to increase the metabolism of Protamone or endogenous thyroxine in the rat(13,15). The antibiotics appeared to exert a partial or complete protective action on the thymus gland against atrophy by the Protamone. It is interesting that Pentz *et al.*(16) also reported that liver and vit. B_{12} partially counteracted the loss in thymus weight of thyroid-fed rats having greatly hypertrophied adrenal glands. They suggested that the protective effects of the liver and vit. B_{12} were exerted directly on the thymus gland.

Summary. (1) Three crystalline antibiotics were fed to 100 male and female rats to determine whether they could counteract the retardation of growth induced by feeding thyrotoxic amounts of Protamone. On a ration adequate in vit. B_{12}, penicillin or neomycin but not streptomycin were completely effective in overcoming the growth-inhibition, while on a ration deficient in vit. B_{12}, all 3 antibiotics were only partially effective. On the deficient ration, the antibiotics reduced the survival rate of the hyperthyroid rats. (2) At least part, if not all, of the effectiveness of the antibiotics in counteracting growth retardation could be attributed to the increase in food consumption and to an increased efficiency in converting food into body weight gains. The antibiotics did not alter the 74% increase in basal oxygen consumption induced by the Protamone, nor did they change the action of the latter on adrenal and spleen weight. Thymus atrophy was partially or completely prevented by the antibiotics.

Received May 21, 1951. P.S.E.B.M., 1951, v77.

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Experimental Pyelonephritis and Hypertension in Rats.* (18919)

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The occurrence of chronic and healed pyelonephritis and the development of arterial hypertension have been shown to be correlated by analysis of clinical material(1,2). Experiments with animals have contributed information concerning the pathogenesis of chronic pyelonephritis(3). To our knowledge the occurrence of arterial hypertension in animals with experimentally induced pyelonephritis has not been demonstrated. Heretofore rabbits have usually been employed in the experimental study of pyelonephritis. The technical advantages offered by the size of these animals are offset by considerations of economy and space. Therefore, a smaller animal was chosen for this study. The use of rats seemed feasible because pyelonephritis develops spontaneously in them(4). Furthermore, rats have been shown to develop hypertension under several experimental conditions(5).

The purposes of this investigation were (1) to develop a technic for the production of pyelonephritis in the rat; (2) to ascertain what changes occur in the arterial blood pressure of rats with experimentally induced pyelonephritis.

Methods. Sixteen white rats weighing about 250 g were used in these experiments. In principle the technic of producing pyelonephritis was similar to that used by Mallory(3). An incision was made in the left flank of a rat under anesthesia. The left ureter was partially mobilized, maintained intact, and a "U" shaped segment passed through the incision in the flank. This segment was maintained in position by a 1.5 cm length of wire passed through the "U" and allowed to rest against the outer muscle layer of the abdominal wall. The muscles were then sutured loosely about the ureter and the skin closed with wound clips. Twenty-four hours or more after the procedure a suspension of viable *Escherichia coli*† was injected into the vein of the tail in a volume of 1 cc. The number of viable organisms injected was determined by a standard counting technic. The "systolic" arterial pressure of each animal was determined by the method of Kersten *et al.*(6) on several occasions preceding and following operation and infection. In a few a Hamilton optical manometer was employed. In some cases examination of the urinary sediment and culture were made. At intervals the animals were sacrificed and examined. Microscopic sections of the kidneys were made. The infected kidneys were cultured for bacteria. In two instances the ligature was removed before the injection of the bacteria.

Results. The results are shown in Table I. Five animals received a dose of viable *E. coli* in excess of 3.0×10^9 organisms. Of these 4 died within 4 days after injection without hypertension developing. Three were found to have definite evidence of acute pyelonephritis as well as early hydronephrosis and hydroureter of the experimentally obstructed kidney. Another (rat 2) had early hydronephrosis of the kidney operated on but little evidence of pyelonephritis. One (rat 1) survived and at autopsy 127 days later was

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* Under a grant-in-aid from the National Heart Institute, U. S. Public Health Service.

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TABLE I. Experimental Pyelonephritis in Rats.

		Autopsy performed			Blood pressure (mm Hg)		Autopsy findings, left kidney					
								Microscopic				
	Rat No.	Days after infection	Days after operation	Dose *E. coli* i.v. × 10^9	Pre op.	Ante-mortem	Gross	Culture	Pyelonephritis	Vascular disease in both	Bacilluria, days after infection	Remarks
Hypertensive	7	41	46	1.6	101	167	Hn. Hu.	+	—	—		Died
	13	48	51	1.9	—	180 (207/172)*	” ”	+	+	+	3	
	14	44	47	.55	—	172 (159/121)*	” ”	+	+	+	3	
	8	104	110	1.6	98	185	Pyn. Pyu.	+	+ Pyn.	+	18	
	12	104	112	1.6	107	159	” ”	+	+ Pyn.	+	18	
	9	106	112	1.6	109	154	Contracted	0	+	+		
	10	106	112	1.6	100	153	”	0	+	+	18	
	15	48	51	2.9	—	168 (181/137)*	”	+	+	+	3	
	1	127	133	18	120	168	1 scar	0	1 area	+	35	
Normotensive	2	4	12	18	110	110	Hn. Hu.	+	±	0	3	Died
	3	1	5	5	115	92	” ”	+	+	—		”
	4	1	5	5	115	99	” ”	+	+	0		”
	6	1	9	5.2	110	103	” ”	+	—	—		”
	16	48	51	2.9	—	100 (77/49)*	N	0	0	0	3	L.R. day 2†
	17	44	47	1.9	—	108 (75/41)*	N	0	0	0	3	” ” ”
	11	—	112	None	113	110	N	0	N	—		” ” ”

* Hamilton manometric reading under anaesthesia.
† L.R. = ligature removed.
Hn. = hydronephrosis. Hu. = hydroureter. Pyn. = pyonephrosis. Pyu. = pyoureter. N = normal.
— = not examined.

found to have no hydronephrosis but an area of pyelonephritis in that kidney which had been subjected to surgery.

Three animals (rats 9, 10, and 15) given smaller doses of *E. coli* were found to have very scarred and contracted left kidneys. The blood supply of these kidneys appeared not altered from the normal and evidence of hydronephrosis and hydroureter was not present. In two animals (rats 9 and 10) the infection was apparently "healed" as cultures of the affected kidneys grew out no organisms. Microscopic examination of these specimens revealed marked destruction of renal tissue, scarring, and inflammatory cellular infiltration. "U" shaped wedges of surprisingly normal appearing glomeruli persisted in each left kidney. The contralateral kidneys were essentially normal except for vascular changes. The blood pressure of these animals was consistently elevated above control levels.

Five animals which survived infection with less than 2.0 × 10^9 viable *E. coli* were sacrificed 44 to 104 days following infection. Two (rats 8 and 12) displayed a striking pyonephrosis and pyoureter on the left side. Microscopic examination of the pyonephrotic kidneys revealed advanced destruction of the renal parenchyma, scarring, and cellular infiltrations of inflammatory character. The only remaining structures recognizable as renal tissues were a few intact glomeruli. Except for microscopic vascular changes the contralateral kidneys were within normal limits. Three (rats 7, 13 and 14) developed moderate to marked hydronephrosis and hydroureter with infection in the renal parenchyma; cultures grew out *E. coli*. The blood pressures of these animals were consistently elevated above the control levels.

In two cases (rats 16 and 17) the wire retaining ligature was removed before the injection of *E. coli* was made; in neither case did pyelonephritis or hypertension develop. In another (rat 11) the operation alone was performed without subsequently injecting *E. coli*.

The wire retaining ligature was removed two days after operation. Hypertension did not develop and at autopsy the left kidney was within normal limits.

Vascular changes in the right unoperated kidney were observed in the animals with hypertension of several weeks' duration. This was usually less in degree from that observed in the infected kidney, but was easily demonstrable. In only one animal was it marked (rat 1).

Discussion. There were 3 findings of interest in these experiments. First, hypertension of some degree invariably accompanied chronic unilateral pyelonephritis in the rats surviving the infection. Hypertension has been induced by unilateral hydronephrosis alone(5) when the kidney was not totally destroyed, but unfortunately superimposed infection was not ruled out. Secondly, in some instances contracted, fibrotic, pyelonephritic kidneys showing no hydronephrosis resulted from partial ureteral obstruction and infection. Thirdly, only the kidney in which there was ureteral or pelvic stasis became infected, a circumstance which might have been predicted(3). In addition, vascular changes in the opposite kidney were seen in those animals which developed hypertension, also a predictable finding(7).

The method used was simple and readily applied. Further studies on prevention or treatment of the pyelonephritis so induced should be rewarding.

Summary. Unilateral renal diseases resembling hydronephrosis, pyonephrosis, or pyelonephritis were induced in rats by partial ureteral constriction and intravenous injection of *E. coli.* Arterial hypertension developed when chronic unilateral renal infection was produced.

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Received May 25, 1951. P.S.E.B.M., 1951, v77.

A Technic for Measurement of Radioactive Iodine (I-131) Uptake by the Human Thyroid Gland. (18920)

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In the study of the uptake of iodine (I-127) by the thyroid, the most practical tracer is the radioactive isotope of iodine (I-131). This isotope has a half life of 8 days and emits 2 types of radiation, beta particles and gamma rays. The maximum energy of the emitted betas is of the order of 600 Kev, while the gammas are emitted with a maximum energy of 723 Kev(1). The conventional Geiger-Muller tube is 90% efficient in measuring beta radiation and only 2% efficient for the gamma radiation. This means that of the incident radiation, 90% of the beta radiation and 2% of the gamma radiation will be recorded. The tube used in our work is a bismuth cathode, Geiger-Muller tube, Radiation Counter Laboratories, Mark 1, Model 12, used axially like an end window counter. The tube has a high gamma efficiency and a low beta efficiency. In an actual comparative experiment using I-131 the bismuth cathode tube showed a count rate of about 7.5 times greater than the conventional end window tube over the same sample. To attain the same counting geometry, the end window tube must be recessed about 1 inch into the lead shield. With this change in tube distance, the bismuth tube count rate is 11 times greater. With the beta radiation filtered out by appropriate aluminum absorbers, the ratio of count rates increases to approximately 16 times. Since most of the beta radiation is

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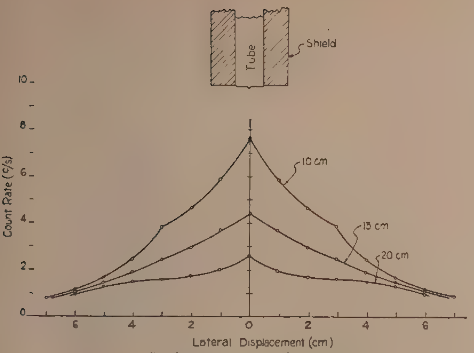

FIG. 1. Point source displacement.

absorbed by the intervening tissue in the *in vivo* measurement of iodine uptake by the thyroid, the latter case is the closest approximation to actual conditions.

Because of the predominance of gamma type radiation in ambient cosmic rays, an increase in the background count rate for the bismuth tube is twice that of the mica window tube. Even with this increase in background there is a 10 to 12 times increase in sensitivity of the tube.

To determine the optimum tube-to-patient counting distance, the directional characteristics of the shielded bismuth tube and the decrease in counting rate with distance were studied. Using a point source of radiation, it was found that at a distance of 15 cm and a lateral displacement of 4.3 cm from the axis of the tube, the counting rate dropped to 50% of the axial value (Fig. 1). That is to say, if all of the radioactivity were concentrated at a point 4.3 cm from the axis, only 50% of the radiation measured at the axis would be recorded. The point source is of course, the worst possible distribution. On the basis of these dimensions we can see that 15 cm is the minimum distance that will give adequate coverage for the "average gland".

Experiments with 2 vials containing equal quantities of I-131 showed that the decrease with this lateral displacement was more of the order of 20%, or, 80% of the radiation was recorded. In this experiment the vials were spread and counted at various distances, symmetrical to the axis of the tube. This is much more nearly the case for the distribution of radioactive iodine in an actual gland (Fig. 2).

With a normal uptake from a 50 uC tracer dose, the counting rate at 20 cm is only 6 to 8 times greater than the background rate. For a counting interval of 6400 counts the statistical error of the Geiger-Muller tube will be of the order of 1%, based on the premise that the counting rate is 10 times greater than the background rate.

Thus, the boundaries are presented. The minimum counting distance, 15 cm, is determined by the directional characteristics of the tube and the maximum counting distance, less than 20 cm, is determined by the limitations of statistical accuracy. Interpolation between these two factors gives an optimum distance of 17 cm, which yields a high enough count rate and an effective counting area at the thyroid of 9.92 cm or about 4 inches in diameter.

It has been stated that the maximum tracer dose of I-131 which will have no deleterious effects on a normal gland is 200 uC per year (2). The minimum efficient tracer dose for the mica-window tube is 100 uC whereas with the bismuth tube, 50 uC is quite efficiently measured.

On this basis, tracer studies are repeatable at intervals of three months as compared with the 6 month interval demanded by the mica-window tubes. Because of the false negatives caused by recent ingestion of such iodine blocking agents as Lugol's solution, propyl-

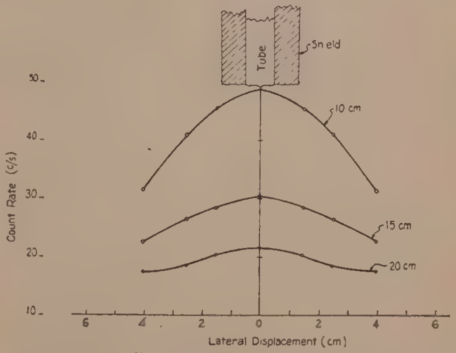

FIG. 2. Vial displacement.

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thiouracil, and various other iodine containing preparations, the shorter interval is highly desirable.

The reference sample diluted to constant volume is placed in a small saline bottle and counted at the same distance of 17 cm. The percentage uptake in the gland is represented by:

$$Up \frac{(Cg \times 100)}{(Cs)} F_G.$$

Where Up denotes the percentage uptake, Cg is the count rate over the gland, Cs is the count rate over the standard sample, and F_G is the geometrical factor.

This geometrical factor is a function of the ratio of the solid angles presented by the gland and the reference sample. If the reference sample is standardized, this factor reduces to a function of the variation of the solid angle presented by the gland. Consequently this factor is a function of the variation in gland size. Standardizing on the basis of the "average gland", this F_G will be greater than one for all glands larger than "average" and so on. However, reconsidering the distributive picture of the directional characteristics of the tube, it is readily seen that no serious error will be introduced by assuming this factor equal to one.

Summary. It has been found that with the bismuth cathode tube, a 50 uC dose can be used efficiently, and that a tube-to-patient distance of 17 cm will cover a sufficiently large area to make variations in gland size unimportant to the accuracy of the test.

Received May 28, 1951. P.S.E.B.M., 1951, v77.

Mandelic Acid Antagonism to Phenyllactic Acid Utilization in *L. casei*.*

(18921)

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In the course of experiments designed to develop microbiological methods for the determination of specific aromatic acids in urine, *L. casei* was found to utilize phenyllactic acid in an otherwise complete but phenylalanine-free medium. This observation has recently been made also in other laboratories(1,2). The present report concerns the growth inhibitory activity of certain structural analogues of phenyllactic acid on this organism.

Methods. Our microbiological assay procedures, media, cultures, inocula, etc., have been described elsewhere(3). DL-Phenyllactic acid was prepared by the methods of Erlenmeyer and Lipp(4), and Fischer and Zemplén(5). Both products when recrystallized twice from carbon tetrachloride melted at 95°C and gave identical growth curves. Melting points of 95-96 and 96-97(6), 97(7), and 97.5°C(2) have been reported for DL-phenyllactic acid.

The growth stimulating effect of phenyllactic acid, phenylalanine, and the various ana-

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* This investigation was supported (in part) by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service, and a grant-in-aid from the Bressler Alumni Research Fund.

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TABLE I. Growth Promoting Activity of Phenyllactic Acid Analogues on *L. casei.*

Compound studied	Quantity of analogue (μg/tube) 0	2.5	5	10	20	40
	Titration values as ml 0.02 N NaOH					
Phenylacetic acid*	1	.9	1	1	.8	1.1
trans-Cinnamic acid†	1	.8	1	1	1	.9
Hydrocinnamic acid*	.9	.9	1	1	1.1	1
trans-o-Coumaric acid‡	1	1.1	1	.8	1.1	1
DL-mandelic acid*	1	.8	1.1	1	1	1.1
p-Hydroxyphenylacetic acid*	1	1	1.1	.9	.8	1
p-Hydroxybenzoic acid†	1.1	.9	1	1	.9	1
β-Phenylisoserine§ (m.p. 231°C)	.2	.4	.3	.2	.2	.2
β-Phenylisoserine§ (m.p. 265°C)	.2	.3	.2	.3	.3	.2
DL-Phenylalanine‖	.8	1.7	3	5.3	7.8	—
DL-Phenyllactic acid	.8	3	5.2	7.8	9.2	—

* Bios Laboratories, Inc., New York City.

† Eastman Kodak Co., Rochester, N. Y.

‡ Synthesized (m.p. 207-208.5°C).

§ Two unidentified isomers synthesized by Dr. Robert Ellin of the School of Pharmacy. Although each isomer could consist of a mixture of two structural forms, no attempt at separation was made. Fourneau and Billeter(7) prepared similar isomers melting at 230-232 and 275-285°C, respectively.

‖ Merck and Co., Inc., Rahway, N. J.

TABLE II. Effect of Analogues on Utilization of Phenyllactic Acid by *L. casei.*

Analogue studied	Phenyllactic acid, μg/tube	Quantity of analogue required (μg/tube) For first indication of inhibition	For complete inhibition	Max quantity of analogue tested
Phenylacetic acid	5	5000	*	8000
trans-Cinnamic acid	5	*	*	400
Hydrocinnamic acid	5	*	*	6000
trans-o-Coumaric acid	5	*	*	250
DL-Mandelic acid	5	500	5000	50000
p-Hydroxyphenylacetic acid	5	500	*	20000
p-Hydroxybenzoic acid	5	2000	*	2000
β-Phenylisoserine (m.p. 230-232°C)	5	*	*	10000
β-Phenylisoserine (m.p. 265°C)	5	*	*	10000

* This value could not be ascertained definitely due to the relative inactivity and/or insolubility of the compound under investigation.

logues was determined by adding appropriate quantities to assay tubes containing 1 ml of the phenylalanine-free basal medium. The contents of each tube were adjusted to pH 6.9, diluted to 3 ml, autoclaved at 18 pounds steam pressure for 10 minutes, cooled, inoculated, and incubated at 34°C for about 72 hours. The acidity produced was titrated with 0.02 N sodium hydroxide to pH 6.9. The inhibitory activity of certain analogues on phenyllactic acid utilization was studied in a similar manner.

Results. The data in Table I show that the various phenyllactic acid analogues were not available to *L. casei.* However, both isomers of DL-phenyllactic acid appeared to be utilized when in the proper concentration. An equal response to D- and to L-phenyllactic acids by *L. casei*(2) and by *L. arabinosis*(1) have been obtained in other laboratories. We found that *L. casei* was not able to convert phenyllactic acid into tyrosine, since growth was not obtained on a medium free of both phenylalanine and tyrosine.

The data in Table II show that DL-mandelic acid is a potent inhibitor of phenyllactic acid utilization as are p-hydroxyphenylacetic acid and p-hydroxybenzoic acid to a lesser degree. Decisive results were not obtained with some of the other analogues due to their relative inactivity and/or insolubility. It is interesting to note that the two mixed isomers of β-phenylisoserine are inert since DL-phenylserine inhibits competitively the func-

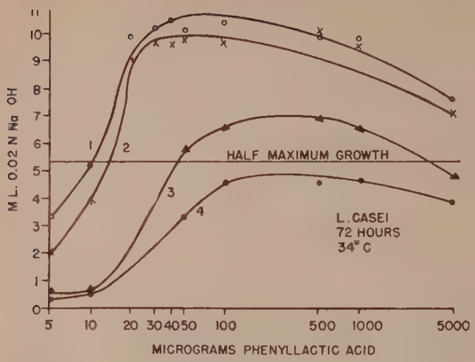

FIG. 1. Effect of DL-mandelic acid on DL-phenyllactic acid utilization. The abscissa is in log scale. Curve 1, 0; curve 2, 1,000; curve 3, 5,000; curve 4, 10,000 μg mandelic acid, respectively.

tioning of phenylalanine in *L. arabinosis*(8).

The good growth response to increments of DL-phenylalanine ranging from 0 to 10,000 μg, even in the presence of 0 to 10,000 μg of mandelic acid per assay tube (data omitted) indicates that mandelic acid *per se* was not toxic to *L. casei* and did not interfere appreciably with the microbial utilization of phenylalanine. The potency of this inhibitor appears to rest primarily on its ability to prevent the enzymatic conversion of phenyllactic acid to phenylalanine.

A more precise examination of the mandelic acid-phenyllactic acid antagonism was made by adding 0, 500, 1,000 5,000, and 10,000 μg of mandelic acid, respectively, to assay tubes each containing 1 ml of phenylalanine-free medium and graded quantities of phenyllactic acid ranging from 0 to 5,000 μg. The assay was carried out in the usual manner and curves were then constructed from the titration data which relate concentration of inhibitor to the amount of growth. A typical response is illustrated by Fig. 1. The data show that phenyllactic acid can reverse the toxicity of mandelic acid to *L. casei*. However, complete reversal was not obtained. While half maximum growth was not achieved in the presence of 10,000 μg of inhibitor, regardless of the amount of phenyllactic acid added, in a second experiment somewhat higher titration values were obtained, namely 5.8, 6.4, and 5.4 ml of 0.02 N sodium hydroxide upon the addition of 100, 500, and 1,000 μg of phenyllactic acid, respectively, to 10,000 μg of mandelic acid. Apparently large quantities of phenyllactic acid are somewhat toxic *per se*.

An examination of the curves in Fig. 1 and other data (omitted) indicates that the inhibitory action of mandelic acid is not competitive over a wide range of nutrilite to inhibitor under our experimental conditions. In a similar but more detailed experiment, the addition of 1,000, 2,000, 3,000, 4,000, and 5,000 μg of mandelic acid to a series of assay tubes containing the basal medium and increments of phenyllactic acid ranging from 0 to 500 μg produced half maximum growth in the presence of 8, 10.5, 15, 20, and 30 μg of phenyllactic acid which yielded inhibition indices of 125, 190, 200, 200, and 167, respectively. Hence, within a narrow range, the inhibitory action of mandelic acid appeared to be competitive.

In view of the widespread use of mandelic acid as a urinary antiseptic, its potency as a metabolic antagonist appears most interesting.

Summary. The growth stimulatory and inhibitory action of several analogues of phenyllactic acid on *L. casei* were studied. DL-mandelic acid was found to be a potent inhibitor of phenyllactic acid utilization by this organism. The action was not competitive, except over a narrow range of inhibitor to nutrilite. Mandelic acid did not interfere with the functioning of phenylalanine.

Received May 28, 1951. P.S.E.B.M., 1951, v77.

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Effect of Alloxan Administration on Liver Nucleoproteins.* (18922)

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Evidence has been accumulating that the desoxyribose nucleic acid (DNA) content of cells is approximately constant in all nuclei of any one species(1,2). It has been reported by Cunningham, Griffin, and Luck(3) that, although an increase in content based on whole tissue occurs in many tumors and in certain tissues following carcinogen administration, nuclei isolated by the Dounce method show no such increase. In the present study liver tissue of alloxan diabetic rats showed not only a significant increase per unit of wet or dry tissue, but also an absolute increase per cell nucleus.

Methods. Rockland Farms male white rats, (180 to 250 g) maintained on Purina Dog Chow and water *ad libitum* were used in these experiments. The animals were fasted 48 hours prior to subcutaneous administration of 175 mg/kg of alloxan-monohydrate (Eastman Kodak Co.) and were sacrificed at intervals up to 6 days following treatment. Liver samples (350-500 mg) taken for nucleoprotein determinations were rapidly weighed and frozen. The nucleic acids were extracted and determined as described by Schmidt and Tannhauser(4). The data obtained using normal rat liver correlated closely with those reported by various authors employing this method(5). Glycogen determinations were made according to the method of Good, Kramer and Somogyi(6). A modification of Payne's method(7) was employed for fat analysis. Tissues for cytochemical study were placed in Carnoy's acetic alcohol (1:3) and cytochemical analyses were performed, utilizing microspectrophotometric technics(1,8-13).

Sections to be stained by the Feulgen nucleal method were cut at 10 μ. All preparations were hydrolyzed simultaneously in 1 N HCl at 60°C for 12 minutes(10,11), then stained for 1 hour in the Schiff reagent(14). Unhydrolyzed control sections, one for each animal, were stained simultaneously in the same reagent. For studying the effect of treatment on the histone and non-histone protein fractions of the cell, 10 μ sections were stained by means of the Millon reaction(8). Ultraviolet absorption measurements of nuclei and cores of cytoplasm (4 μ in diameter) were made on sections 3 μ thick. All absorption measurements were made with a Photovolt electronic multiplier photometer (Mod. 512). For measurement of the amount of Feulgen dye bound by the nuclei, the desired wavelength of light was isolated from a G. E. AH4 mercury lamp and a Farrand interference filter with a transmission peak of 560 mμ; for measuring the intensity of the Millon reaction in nuclei and cores of cytoplasm a Corning

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* This investigation was supported by the National Institutes of Health, Public Health Service and Hendricks Research Fund. The authors wish to acknowledge the technical assistance of Mrs. Marjorie Stofel, Mrs. James Frazer and Miss Helen Braunfelds.

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TABLE I. Nucleoprotein Changes Occurring in Alloxan Treated Diabetic Rats.

Exp	Treatment	No. of rats	No. of observations	Days after treatment	Liver nucleic acids, mg P					
					Per 100 g wet wt		Per 100 g dry wt		Per 100 g protein dry wt	
					DNA ± SE*	RNA ± SE	DNA ± SE	RNA ± SE	DNA ± SE	RNA ± SE
1	Controls (saline)	28	56		20.15 ± .51	102.9 ± 1.18	68.17 ± 4.13	349.4 ± 2.40	100.3 ± 3.9	513.63 ± 6.74
Nov. 1950	Alloxan†	14	28	4	27.4 ± .69	108.9 ± 2.00	95.27 ± 2.41	344.2 ± 5.15	133.23 ± 4.33	482.6 ± 12.17
2	Controls (saline)	4	8		17.2 ± .55	106.6 ± 4.30				
Apr. 1951	Alloxan	3	6	1	21.2 ± .84	123.4 ± 2.30				
	,,	9	18	3	21.1 ± .90	112.8 ± 2.36				
	,,	7	14	5-6	22.8 ± 1.15	103.6 ± 2.88				

* $\text{SE} = \frac{\text{SD}}{\sqrt{n}}$.

† Alloxan diabetic rats had blood sugar values ranging from 150 to 885 with a mean of approximately 400 mg %.

glass filter having a maximum transmission at 365 mμ was used. For the ultraviolet absorption measurements, the 2537 Å line was isolated from a Hanovia mercury lamp, by means of a Cooke double prism quartz monochromator. Throughout this series of measurements, care was taken to choose only round nuclei having approximately the same diameter (about 7 μ).

Results. DNA per unit of whole liver was increased in the alloxan treated animals (Table I). When calculated on the basis of dry weight or total protein this difference was still apparent. The liver lipid and glycogen content of the diabetic rats did not differ significantly from that of the control animals. Liver lipid in the untreated rats was 5.59 ± 0.15%, in the alloxan diabetic rats 5.13 ± 0.145%; corresponding values for liver glycogen were 6.36 ± 0.23% and 5.9 ± 0.25%, respectively. Each figure represents a mean with its standard error. Their P values are > .05 and > .1, respectively. The increase in DNA was confirmed by cytochemical analysis (Table II).

Tissue taken 4 days after alloxan administration showed no deviation in total RNA from that of the controls. Ultraviolet absorption measurements on the cytological preparations indicated no change in cytoplasmic RNA. Though no estimations of nuclear RNA have been made, indirect evidence that it, as well as DNA, is increased after alloxan is given in Table II. Since the percentage increase in total nucleic acid is greater than that of DNA, the difference is presumably due to RNA.

An increase in total protein content of the nucleus was indicated by the increase in intensity of the trichloracetic acid Millon reaction. The measured extinction value for a control animal (No. 211) was 0.422 ± 0.006, the corresponding value for a treated animal (No. 204) 0.482 ± 0.006. In the cytoplasm, however, no such increase was observed. Since the ratio of trichloractic acid Millon extinction to Feulgen extinction remained constant for both control and experimental tissues, the protein increased in proportion to the DNA. That this protein in-

TABLE II. DNA and Total Nucleoprotein Changes Occurring in Alloxan Treated Diabetic Rats.

Treatment	Animal No.	DNA‡	Feulgen nucleal† reaction for DNA, $\overline{E}_{560} \pm SE$	Total nucleic acid† ultraviolet absorption (nucleus), $\overline{E}_{2537} \pm SE$
Alloxan*	200	27.2	.336 ± .004	.339 ± .006
	204	30.5	.342 ± .005	.323 ± .008
	198	28.6	.335 ± .005	.329 ± .006
Saline	211	18.9	.288 ± .003	.230 ± .006
	212	19.6	.290 ± .003	.243 ± .005
	213	22.7	.295 ± .004	.248 ± .004

* Animals were sacrificed 4 days after alloxan administration.
† Each figure represents an average extinction value of 15 to 25 nuclei.
‡ mg P/100 g of liver (wet wt).

crease was due primarily to an increase in the histone fraction was indicated by the observation that removal of histone from both treated and control sections eliminated the differences in the intensity of color produced by the standard Millon reaction.

The increase in DNA could have resulted from either an increase in the number of nuclei present per unit of tissue or from a change in nuclear volume. The cytochemical studies exclude the first possibility; the second was excluded by measurements which showed that no change in the nuclear volume resulted from the alloxan administration. It should be emphasized that the determination of DNA is expressed as mg/100 g of wet liver weight, whereas the Feulgen and ultraviolet extinction data are expressed as extinction values/nucleus. The data therefore, are not directly comparable, but results obtained with both methods show an increase in DNA.

Discussion. High DNA values were found uniformly on the 4th, 5th and 6th days following alloxan administration. The increases observed were small during the first 3 days. This is consistent with the reports of Brues *et al.*(15) that the DNA turnover, as measured by isotope technics, is relatively slow. Rerabek(16) reported that alloxan administration to rats produced an increase in liver RNA but no change in DNA. It is not possible to compare Rerabek's results with ours since he gives no evidence to indicate that his animals were diabetic, nor does he state what interval elapsed between alloxan administration and removal of the tissue for analysis.

The relationship of the DNA increase to the diabetic state is being studied. The data presented do not indicate whether the effect noted is specifically due to the action of alloxan on hepatic tissue or arises from the destruction of beta cells in the islands of Langerhans. Possibly this will be clarified by experiments in progress on rats made diabetic by partial pancreatectomy. Since the DNA increase cannot be correlated directly with blood sugar levels, it seems reasonable to assume that it is a direct effect of alloxan.

Summary. Administration of alloxan to white rats causes an increase in the DNA and the histone of liver nuclei. Direct measurements indicate no change in cytoplasmic RNA, but, indirect evidence suggests an increase in nuclear RNA.

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Received May 21, 1951. P.S.E.B.M., 1951, v77.

Comparison of Leukocyte Changes Produced by Pyrogens and by Anaphylaxis in the Guinea Pig.* (18923)

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It is well known that in human beings and animals, the injection of bacterial pyrogens or endotoxins produces a precipitous fall in circulating leukocytes at the expense of the polymorphonuclear cells; this initial reduction is followed by a polymorphonuclear leukocytosis within a few hours(1). Many investigators have also reported that anaphylactic shock in animals is accompanied by an initial reduction in peripheral polymorphonuclear cells followed shortly by leukocytosis if animals survive the acute episode(2). Although the symptoms and physiologic changes produced by pyrogens and those accompanying anaphylaxis are easily distinguished and generally recognized as separate entities, the hematologic changes are not. Current hematology texts lump together as causes of sudden leukopenia "anaphylactoid shock and early stages of reaction to foreign protein"(3), "anaphylactic shock due to introduction of a foreign protein" or "acute protein shock such as is caused by the intravenous injection of typhoid vaccine"(4).

It is the purpose of the present report to show that the variations in circulating leukocytes which accompany anaphylaxis in the guinea pig are not due to pyrogen contamination and to point out that a difference in behavior of the circulating eosinophils makes it possible to distinguish between the two types of reaction in this animal.

Methods. Male guinea pigs weighing 250 to 350 g were used. Diet consisted of laboratory pellets with added green vegetables twice weekly. The following antigens were used: normal horse serum, diphtheria antitoxin (Squibb), and crystalline ovalbumin. Pyrogen preparations were *Escherichia coli* and typhoid vaccines containing 1.0 billion organisms per ml and a filtrate of *Pseudomonas aeruginosa* prepared by the method of Welch (5). Intravenous injections were made into the dorsal penile vein. Total leukocyte counts were performed by the usual methods, employing 3% acetic acid with 0.1% added gentian violet as a diluting fluid. Except for a few experiments in which slide smears were made, cover-slip smears stained with Wright's stain were examined for differential counts. Eosinophils were enumerated by a direct chamber method within 5 minutes after collecting blood. The acetone-water-eosin fluid recommended by Thorn *et al.*(6) was found satisfactory. Pipettes for eosinophil counts were shaken 150 times after loading. Blood was collected from fresh cuts made over ear veins. At least 2 control counts were obtained before injection of test materials and post-inoculation counts were taken at varying intervals. All syringes and glassware were sterilized at 170°C for 2 hours to destroy any contaminating pyrogen. 0.9% sodium chloride solution and solutions of antigens were tested at frequent intervals in rabbits and were always pyrogen free.

Effect of anaphylaxis produced by non-pyrogenic antigens upon leukocyte count. Eight animals were given 0.1 ml of diphtheria antitoxin (Squibb) intravenously, total and

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* This work was supported by the Anna H. Hanes Memorial Fund.

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TABLE I. Absolute Polymorphonuclear Leukocyte Counts of Guinea Pigs Given 1 ml Horse Serum Intraperitoneally on 1st and on 21st Experimental Day. Counts 5 min before and 20 min after injection. All figures refer to mean ± standard error.

Day	No. animals	Before injection	After injection
1	8	5536 ± 541	5935 ± 410
21	8	5979 ± 768	990 ± 253

differential counts being done on blood taken 5 minutes before and 5 minutes after injection. No consistent alterations in the number of circulating white cells were noted. The injection of 0.1 ml of antitoxin was repeated 20 days later. Leukocyte counts 5 minutes after injection consistently showed a marked diminution in circulating polymorphonuclear cells. All animals developed typical signs of anaphylaxis after reinjection, 7 of the 8 dying within 10 minutes. The lone survivor was given 0.1 ml of antitoxin intravenously 24 hours later without significant change in leukocyte count. In a similar experiment, 6 animals were given 1.0 mg of crystalline ovalbumin in 1.0 ml of 0.9% sodium chloride solution intravenously; no consistent change in leukocyte count ensued. The intravenous injection of 1.0 mg of ovalbumin was repeated 20 days later. Total leukocyte counts dropped sharply within 5 minutes at the expense of the polymorphonuclear cells. All animals had typical symptoms of anaphylaxis and died within 20 minutes after reinjection. In the third experiment, 8 animals were given 1.0 ml of non-pyrogenic horse serum intraperitoneally, leukocyte counts being performed before injection and 20 minutes after injection. No consistent changes were noted. After 20 days, the intraperitoneal injection of horse serum was repeated. A leukopenia due to disappearance of the neutrophils was noted at 20 minutes, followed by a leukocytosis at 4 hours. Although all animals showed evidence of anaphylaxis with ruffling of fur, dyspnea, etc., none died. Table I shows the polymorphonuclear response in this group of animals. In a fourth experiment, 6 guinea pigs were given 2.0 mg of crystalline ovalbumin intraperitoneally on the first experimental day and this injection was repeated on the 21st day. Leukocytes remained relatively unchanged on the first day, but after sensitization, all animals responded with leukopenia followed by leukocytosis. Table II illustrates the changes in polymorphonuclear cells in this group of animals.

Effect of pyrogenic materials upon leukocyte count. Six animals were given 1.0 ml of *E. coli* vaccine intraperitoneally, total and differential leukocyte counts being done just before injection and at 20 minutes, 1 hour, and 4 hours after injection. The changes in leukocytes followed a pattern entirely similar to those noted during anaphylaxis. In similar experiments, groups of 4 animals received 1.0 ml of typhoid vaccine or 5.0 ml of *Ps. aeruginosa* filtrate intraperitoneally. The leukocyte changes in these animals followed the pattern described above. Table III depicts the changes in neutrophils after pyrogen injection.

Effect of anaphylaxis and pyrogen injection upon circulating eosinophils. Total eosinophil counts were performed upon guinea pigs given 2 injections of 1.0 ml of horse serum intraperitoneally 21 days apart and upon the group given 2.0 mg of ovalbumin intraperitoneally. Two control counts were done before injection with counts at 20 minutes and then hourly intervals up to 4 hours after injection. The first injection of antigen produced no consistent change in circulating eosinophils although there was a tendency for the counts to drop slightly during the 4 hours, perhaps as a result of handling the animals. Reinjection of the antigen produced a striking rise in eosinophils in every animal. This increase was usually noted at about the second hour and was invariably present at 4 hours. The injection of *E. coli* vaccine, typhoid vaccine, or *Ps. aeruginosa* filtrate intraperitoneally produced a sharp drop in circulating eosinophils, at times causing them to disappear entirely by the fourth hour. Tables IV and V contrast the effects of anaphylaxis and pyrogens upon circulating eosinophils.

Discussion. The experiments reported here confirm the observations of others that anaphylactic shock is accompanied by a rapidly developing leukopenia followed by a

TABLE II. Absolute Polymorphonuclear Leukocyte Counts of Guinea Pigs Given 2 mg Ovalbumin Intraperitoneally on 1st and on 21st Experimental Day. All figures refer to mean ± standard error.

Day	No. animals	Before injection	20 min	1 hr	4 hr
1	6	5313 ± 820	5576 ± 564	5942 ± 761	5461 ± 684
21	6	5135 ± 524	1368 ± 314	5351 ± 1179	19470 ± 1757

TABLE III. Absolute Polymorphonuclear Leukocyte Counts of Guinea Pigs After Injection of *E. coli* or Typhoid Vaccine. Figures refer to mean ± standard error.

No. animals	Before injection	20 min	1 hr	4 hr
14	5170 ± 553	1122 ± 650	5096 ± 860	18769 ± 1748

TABLE IV. Circulating Eosinophils After Injection of Pyrogen-Free Antigens on 1st and 21st Experimental Day. Figures refer to mean ± standard error.

Day	No.	Before injection		20 min	1 hr	2 hr	3 hr	4 hr
1	14	52 ± 13	47 ± 12	54 ± 14	52 ± 14	53 ± 18	44 ± 14	50 ± 13
21	14	77 ± 17	72 ± 17	70 ± 12	80 ± 13	122 ± 25	259 ± 13	360 ± 12

TABLE V. Circulating Eosinophils in Guinea Pigs After Injection of *E. coli* or Typhoid Vaccine. Figures refer to mean ± standard error.

No.	Before injection		20 min	1 hr	2 hr	3 hr	4 hr
14	73 ± 11	74 ± 10	74 ± 9	79 ± 6	59 ± 4	23 ± 3	14 ± 3

leukocytosis. This type of hematologic response is also characteristic of the reaction to injection of bacterial pyrogens. However, since pyrogen free antigenic materials produced this response in sensitized animals, these changes are not due to pyrogen contamination.

Herrick and, more recently, Campbell(7), using various antigens, have shown that the reinjection of antigen in a sensitized animal results in peripheral eosinophilia. These workers recorded eosinophils in terms of per cent or computed absolute values by the relatively inaccurate method of simultaneous total and differential counts. None of their studies was done with serial counts at brief intervals after injection; for the most part, only single daily counts were recorded. Ishihara(8), however, using a modification of Dunger's original direct counting method, found a marked increase in circulating eosinophils within 1 to 3 hours after administration of a non-fatal shocking dose of antigen to sensitized guinea pigs.

It has been pointed out that the injection of typhoid vaccine in human subjects is followed by eosinopenia, similar to that seen as a result of adrenocortical stimulation(9).

The initial leukopenia followed by polymorphonuclear leukocytosis which occurs after reinjection of an antigen in a sensitized animal is separate and distinct from the hematologic changes after pyrogen injection. These two reactions can be distinguished by the difference in behavior of circulating eosinophils which rise after anaphylaxis and fall after pyrogen injection.

Summary and conclusion. (1) Changes in circulating leukocytes which occur after anaphylaxis have been compared with those after pyrogen injection. Following both of these reactions, there is immediate leukopenia followed by polymorphonuclear leukocytosis. (2) Two types of response, although superficially very similar, are distinguishable by direct eosinophil counts which reveal uniform

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rises with anaphylaxis and uniform falls with the pyrogenic reaction. (3) The mechanism of these hematologic changes is not touched upon in the present study. (4) It is concluded that the leukocyte changes after anaphylaxis are not due to pyrogen contamination.

Received June 19, 1951. P.S.E.B.M., 1951, v77.

Serologically Reactive Material in Spinal Fluid, Blood, and Urine from a Human Case of Cryptococcosis (Torulosis).* (18924)

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The purpose of this paper is to report the occurrence and the serological detection of reactive substances ("soluble antigens") in the spinal fluid, blood, and urine of a patient with cryptococcal meningitis; and also to describe the serological reaction of the capsules of the *Cryptococcus* cells contained in the sediment of the spinal fluid.

Materials and methods. The materials were from a patient with cryptococcal meningitis at Okmulgee, Okla.; a clinical report of the case will be published elsewhere by Dr. McCauley(1). The samples were obtained about 5 weeks after the patient's admission to the hospital and about a week before the fatal outcome; the spinal fluid was taken 2½ days earlier than the blood and the urine.

The spinal fluid was clear in gross appearance and the urine (a first morning sample of about 200 ml) proved normal in routine chemical analysis. *Cryptococcus* cells were present in considerable number in the spinal fluid, but none were demonstrable in either the blood or the urine by culture or by microscopic examination. The samples were transported to New York City for the serological tests.†

The serological tests on the spinal fluid, blood serum, and urine were made with supernatants cleared by centrifugation. In the case of the urine, tests were also made with solutions of alcohol precipitable material, prepared by 3 precipitations with 2.5 volumes of alcohol and extractions of the precipitate with water; the final solution, made in one-fourth the original volume, was water clear and more suitable for serological tests than the original urine. The precipitation test mixtures consisted of equal volumes (0.2 ml) of antigen and of serum; observations were made after 1½ to 2 hours at room temperature, and again after 3 days storage in the icebox. The complement fixation tests were made with similar mixtures of antigen and of serum plus 2 units of complement (0.2 ml); after overnight storage at 5°C followed by a 10 minute period in a 37°C water bath, 0.2 ml of sensitized sheep r.b.c. were added to the mixtures which were then put at 37°C for a one-hour hemolysis test period.

Experimental. A series of dilutions of the patient's fluids were tested for both precipitat-

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* This investigation was aided by a grant from The Louis Livingston Seaman Fund, New York Academy of Medicine and by a fund from the Hayt Foundation, Inc.

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† Merthiolate (0.01%) was added to all of the samples immediately after they were taken from the patient, and an equal volume of 95% alcohol was added to the major portion of the urine. The blood and the urine samples were maintained below 5°C throughout the entire time of transport, but the spinal fluid which was shipped separately by air mail was not packed in ice. Although the merthiolate (0.01%) did not kill all of the cryptococci in the spinal fluid, it was apparently sufficient to have prevented any growth during the transport of the sample, since cultures of the strain isolated failed to grow when large inoculums were tried in broth and in Berkefeld filtered spinal fluid (from other patients) to which either 0.01 or 0.001% merthiolate had been added.

TABLE I. Serological Reactions of Spinal Fluid, Blood Serum and Urine of a Cryptococcosis Patient and of Solutions of Purified *Cryptococcus* Polysaccharides.

	Titres* of the materials in tests against *Cryptococcus* antiserum			
	Precipitation		Complement fixation	
Materials	1:5 serum	1:20 serum	1:40 serum	1:160 serum
Patient's spinal fluid	100	100	400	400
,, blood serum	200	200	800	800
,, urine	U	U	20	10
Control spinal fluids, serums, urines	0	0	0	0
Solutions of *Cryptococcus* polysaccharide, containing 0.01 mg/ml	50	50	400	200

* The titres represent the maximum dilution of the materials which gave positive reactions when mixed with an equal volume of the antiserum. U represents a positive reaction obtained only with undiluted material.

ing and complement-fixing capacities against a potent *Cryptococcus* antiserum and also against a pool of normal rabbit serums and the pre-immunization (normal) bleeding of the rabbit from which the test antiserum had come. Two samples of normal human serum, 10 samples of spinal fluid, and 6 morning samples of urine obtained from patients in the New York Hospital with illnesses other than cryptococcosis, and a solution of alcohol precipitable material prepared from one normal urine were included as controls. For comparison, solutions of 2 different preparations of purified *Cryptococcus* polysaccharides,[‡] each containing 0.01 mg per ml, were tested in the same series of dilutions as those employed with the patient's fluids; one of these polysaccharides had been prepared from the strain which had been employed to produce the antiserum used in the experiment.

The dilutions of the rabbit serums were 1:5 and 1:20 for the precipitation and 1:40 and 1:160 for the complement fixation tests; these dilutions represented, respectively, about 1/64 and 1/16 of the maximum titre of the antiserum in preliminary standardization experiments against solutions of purified *Cryptococcus* polysaccharides. An antiserum sufficiently potent to permit the employment of relatively high serum dilutions for the complement fixation tests was utilized in order to avoid the possibility that normal rabbit serum in low dilution might have some complement-fixing capacity with the patient's serum.

Results. All the fluids from the patient reacted with the *Cryptococcus* antiserum (Table I). In the case of the spinal fluid and blood serum, 1:5 dilutions gave strong flocculation immediately, 1:100 dilutions gave definite clouding within a few minutes, and 1:400 to 1:800 dilutions gave positive complement fixation; in contrast, the control spinal fluids and blood serums from other persons proved entirely negative even when tested undiluted. Further evidence that the amount of reactive material contained in the patient's spinal fluid and blood was relatively great is that both of them precipitated in slightly but definitely higher dilutions than did solutions that contained 0.01 mg per ml of purified *Cryptococcus* polysaccharides. All of the tests with the control rabbit serums were negative and are omitted from the table.

The large amount found in the blood is of particular interest because no *Cryptococcus* cells were demonstrable in the sample either by culture or by microscopic examination. This finding is analogous to the occurrence, in some cases of pneumonia, of pneumococcal reactive substances in blood samples that contain no demonstrable pneumococci(6), and indicates that *Cryptococcus* reactive substances (probably capsular polysaccharide material) may be liberated into the blood from the site or sites of the infection without being accompanied by the fungus cells. The concentration of reactive material in the morning sample of urine was considerably less than that in the blood sample obtained at approximately the same time, which suggests that the major portion of the reactive *Cryptococcus* material contained in the blood of this patient consisted of substances that do not readily pass through the kidney. It is worthy of note that the lower concentration in the urine in comparison to the blood is different from the relationship that occurs in at least some cases of pneumococcal lobar pneumonia(6-8).

[‡] The polysaccharides were kindly supplied by Dr. Edward J. Hehre and coworkers. One was prepared from a culture grown in a synthetic fluid medium(2) and the other from washed *Cryptococcus* cells that had been grown on agar(3).

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The reactive substances in all the fluids of the present patient were relatively heat stable, since boiling for 10 minutes caused no loss in reactivity. Moreover, absorption experiments were made which showed that treatment with solutions of purified *Cryptococcus* capsular polysaccharide‡ removed the capacity of the antiserum to react with the patient's spinal fluid and blood serum. These results furnish evidence of the immunological specificity of the reactions obtained with the patient's fluids and also strongly indicate that the reactive substances represent capsular polysaccharides derived from the fungus with which the patient was infected.

Capsular reactions. The *Cryptococcus* cells from the sediment of the centrifuged spinal fluid gave Quellung-like reactions§ in moist mounts containing antiserum. These reactions of the capsules, (Fig. 1 and 2), are of academic importance because there is no previous evidence in the literature that capsules of *Cryptococcus* from human infection contain serologically reactive constituents. The capsular reactions seem also of some practical importance because the greater visual prominence of the fungus cells in preparations containing a reactive antiserum (Fig. 1) should aid in the microscopic search for *Cryptococcus* cells in materials from patients and in the distinction of the fungus cells from lymphocytes. The capsules also become evident in ordinary India preparations(9) but the serological preparations in our opinion are less likely to be complicated by artefacts. In practice, of course, the Quellung test would be useful only with materials containing sufficient numbers of the fungus cells to be detected microscopically.

The *Cryptococcus* cells in Fig. 1 and 2 had been washed once with salt solution to remove the excess of free soluble antigen. In similar preparations of unwashed cells, long strands and amorphous masses (presumably soluble antigen precipitated by the antiserum) occurred, especially around the clumps of agglutinated cells, which simulated the "reticulated" appearance described by Frisch(10) for the Quellung reaction of some type 3 pneumococcal sputums. The same sort of phenomena was observed also in Quellung tests upon the peritoneal fluids of *Cryptococcus*-infected mice(11).

Comment. The data are of academic importance because they present the first demonstration of the occurrence of serologically reactive *Cryptococcus* substances in body fluids of a human infected with that fungus. The samples tested were obtained at a late and grave stage of the patient's infection and, *a priori*, might be expected to contain larger

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§ The term Quellung-like is used for convenience and also because of the similarity in immunological principle between the reactions of the capsules of *Cryptococcus* and the Quellung reactions of pneumococci and other bacteria. However, as pointed out previously(4), the major part of the visible phenomenon in the case of *Cryptococcus* seems to be due to a change which makes the capsule more readily seen rather than to any great increase in the size of the capsule.

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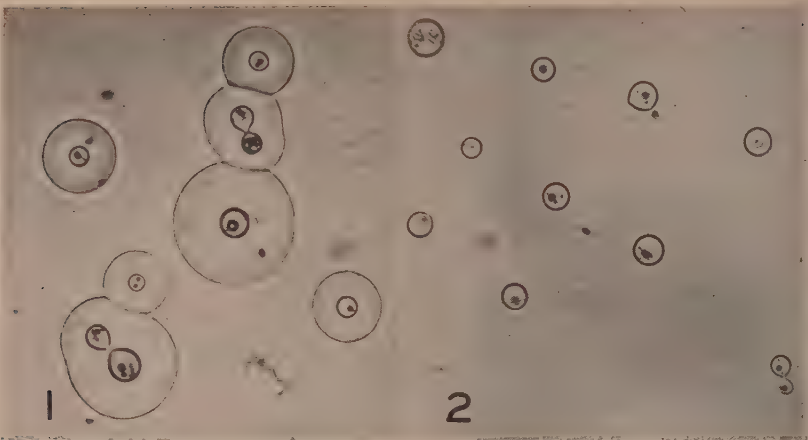

FIG. 1. *Cryptococcus* cells from the sediment of the patient's spinal fluid plus *Cryptococcus* rabbit antiserum. FIG. 2. *Cryptococcus* cells from the same suspension plus normal rabbit serum. Note the prominence and the sharply outlined borders of the capsules in Fig. 1 whereas only faint and equivocal halos are evident in the control mount illustrated in Fig. 2. The magnification of the illustrations is × 560. The cells in Fig. 1, including the capsules which comprised from ⅔ to ¾ of the total measurement, range from about 160 to 300 μ in diameter, but larger cells, some of which were as much as 475 μ in diameter were not uncommon in the sediment from the spinal fluid.

amounts of reactive material than would samples from earlier stages or milder forms of cryptococcal infection. However, the relatively large amounts of the reactive material that occurred in this case and the ease and speed of the serological detection suggest that tests upon body fluids of patients may have some practical importance in the study of human cryptococcosis. That question, however, can be determined only by trials with materials from a reasonably large number of patients. We are now attempting to collect material for that purpose, and would appreciate receiving samples from clinical workers who encounter cases of cryptococcal infection.

Summary. Serologically reactive substances ("soluble antigens") which were readily detectable by precipitation and by complement fixation with *Cryptococcus* antiserum were found in the spinal fluid, blood serum, and urine of a patient with cryptococcal meningitis. Absorption of the antiserum with solutions of purified *Cryptococcus* polysaccharide removed its capacity to react with the fluids of the patient. The reactive substances in the patient's fluids were relatively heat stable and it is probable that they represent capsular polysaccharides of the fungus with which the patient was infected.

Serological reactions of the Quellung type were obtained with the capsules of *Cryptococcus* cells from the centrifuged sediment of the spinal fluid.

Received June 24, 1951. P.S.E.B.M., 1951, v77.

Influence of Female Hormones on Motility of Cat's Uterus and Its Responses to Oxytocics. (18925)

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The uteri of cats chosen at random are frequently quite insensitive to α-hypophamine (Pitocin) and synthetic oxytocics. To overcome this limitation an extended study was

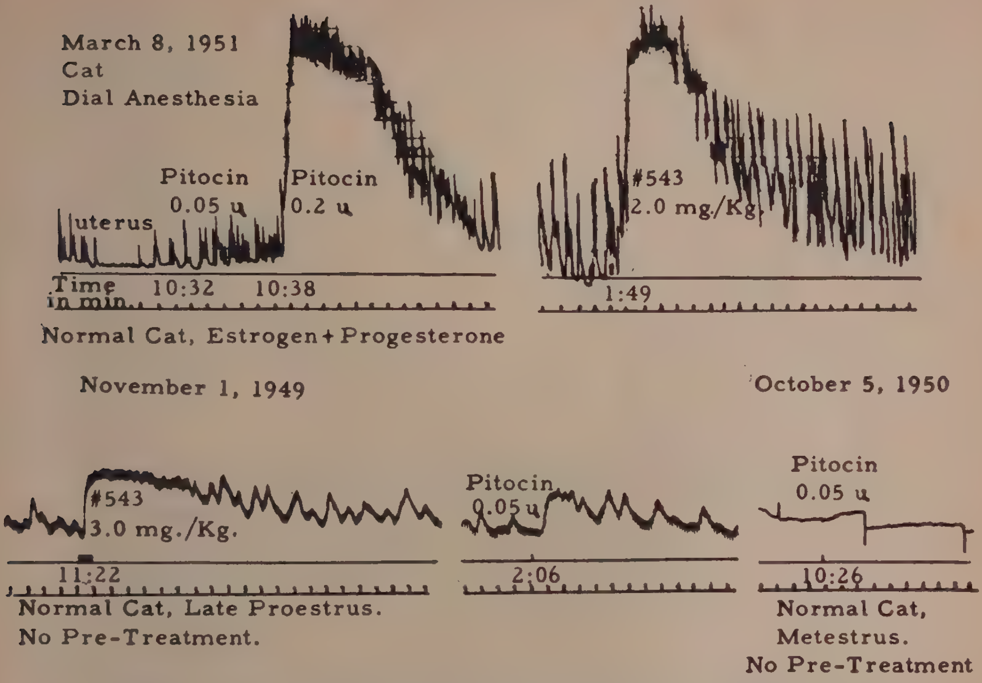

FIG. 1. *Mar. 8.* Response to pitocin and synthetic oxytocics in cat primed with 1.0 mg α-estradiol dipropionate (intramusc) on first and third days and 1.0 mg progesterone (intramusc) on sixth, seventh and eighth days. Experiment on ninth day. Units of pitocin in total dose. Preparation 543 : 2-methyl-6-N-methyl-N-(β-piperidino-ethyl) amino pyridine monohydrochloride. In this, and the subsequent figures, the recordings of blood pressure and respiration have been omitted to conserve space since the changes were not notable.

initiated to determine if suitable hormonal treatment of the cats prior to experimental use could assure uteri responsive to drugs. Wick and Powell(1) earlier reported that priming cats with estrogen increased the responsiveness of the uteri, but cats so treated did not always present active uteri. A method for rendering a uterus responsive to drugs and the experiments upon which it was based, totaling over 200, are described below.

Method. The method of recording the movements of the cat's uterus *in vivo* has been previously described(2). The Trendelenburg modification(3) of filling the belly with Tyrode's solution was employed and a glass tube was snugly fitted into the abdominal opening so the partially withdrawn loop was submerged in the fluid. This yielded longer-lasting preparations than those exposed to air. The following hormonal treatment of cats was found to provide uteri highly responsive to oxytocics over a period of many hours. For 8 consecutive days 0.3 mg of α-estradiol dipropionate was injected intramuscularly; on the last 3 days 1.0 mg of progesterone was also injected. This treatment yielded on the ninth day a uterus extremely reactive to drugs throughout the day. An alternative method that yielded almost as sensitive a uterus and eliminated the need for daily injections was the injection of 1.0 mg of α-estradiol dipropionate on the first and third days and the injection of 2.0 mg of progesterone on the sixth, seventh, and eighth days. The cat was then usable on the ninth day (Fig. 1).

Results of hormonal treatment of juvenile,

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TABLE I. No. of Cats Used in Various Experiments.

	Normal	Progesterone	Estrogen	Estrogen + Progesterone
Normal	25	5	75	44
Pregnant	12	3	3	3
Juvenile	1	3	4	6
Castrate	1	8	5	14

castrated, and pregnant cats. Experiments were performed with juvenile, castrated, and pregnant cats to define more clearly if possible the influences of gestational and estrogenic hormones, given alone or together, on the motility of the uterus and its responsiveness to drugs. Table I summarizes the number of animals used in each group.

A. *Juvenile cats.** Estrogen alone in doses of 1.0 mg on the first and third days yielded a uterus on the ninth day slightly responsive to drugs for a short interval. The same was true for the uterus of cats used on the fifth day which had received four consecutive daily doses of 5.0 mg of progesterone. Juvenile cats given 1.0 mg of estrogen on the first and third days and 5.0 mg of progesterone on the sixth, seventh and eighth days, presented uteri quite sensitive to oxytocics on the ninth day. The uteri of the cats untreated with hormones were entirely unresponsive to all drugs (Fig. 2).

B. *Castrated cats.* In general castrated cats responded like juvenile cats except that such cats given α-estradiol dipropionate alone or in combination with progesterone offered uteri somewhat more responsive to uterotonics. Even this activity, however, did not match that exhibited by the uterus of a responsive normal cat after the injection of oxytocics (Fig. 3).

C. *Pregnant cats.* The uteri of untreated pregnant cats were unpredictable in their behavior. They varied from complete lack of responsiveness to drugs to extreme sensitivity toward them. With several cats in roughly the latter half of pregnancy, not injected with hormones, a single drug injection initiated a hypermotility that continued unabated throughout the day. A similar finding for the uteri of pregnant sheep, *in vitro,* was reported by Ambache and Hammond(4). Attempts to diminish the motility of these pregnant uteri with high doses of antispasmodics were of no avail, an observation in agreement with the work of Diehl and Hundley(5). These variations, as studied in over a dozen untreated pregnant cats, did not seem correlated with the duration of gestation. In pregnant cats estrogen alone, as well as estrogen plus progesterone, initiated an extraordinary motility and sensitivity to synthetic oxytocics, but the responses subsided in time. Cats primed with progesterone alone exhibited greatly prolonged responses of the uterus to synthetic uterotonics (Fig. 4).

Correlation of vaginal cytological studies with subsequent uterine responsiveness to oxytocics. Only the uteri of cats in estrus or proestrus exhibited a responsiveness to oxytocics. Not infrequently even the uteri of cats in these two stages of the estrus cycle proved unreactive. Sometimes cats treated with an estrogen alone exhibited the cytological changes of estrus, but despite that were unresponsive to drugs.

In a considerable series of experiments efforts were made to restore the responsiveness of a uterus that had lost its activity to oxytocics. The drugs so employed included aqueous estrone sulfate, α-estradiol dibenzoate in oil, progesterone in oil, testosterone in oil, glucose, desoxycorticosterone glucoside, choline and vitamin B complex.† The use of the latter two agents was suggested by the report of Peet and Sampson(6) that rats on a choline deficient diet yielded non-motile uteri. Kraatz and Gruber(7) were unable to confirm this observation although they did

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* So adjudged if they did not possess permanent back molars. Histological sections of the uterus were also made.

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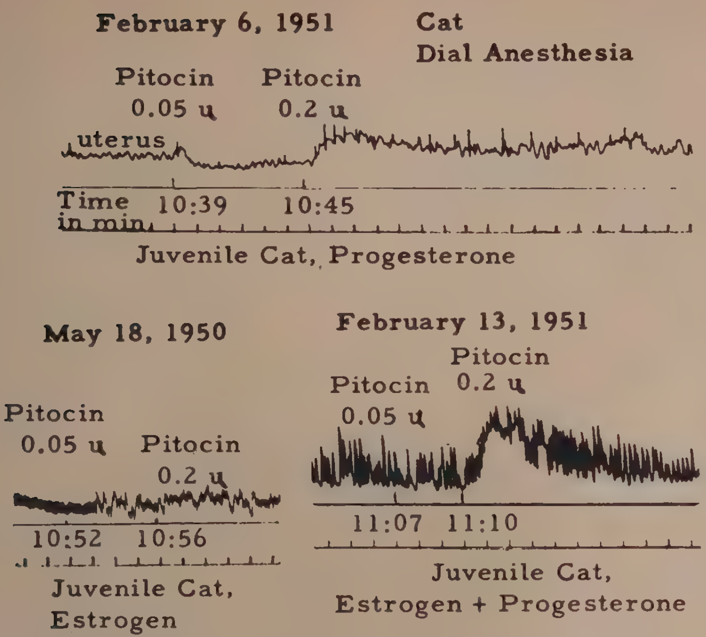

FIG. 2. *Feb. 6.* Units of pitocin in total dose. 5 mg doses of progesterone (intramusc) for 4 consecutive days. Experiment on fifth day. *May 18.* 1 mg α-estradiol dipropionate (intramusc) on first day and third day. Experiment on ninth day. *Feb. 13.* 0.3 mg α-estradiol dipropionate (intramusc) first through eighth day and 5 mg progesterone (intramusc) on days 6, 7 and 8. Experiment on ninth day. Compare with normal cat in late proestrus stage that received no pre-treatment (Fig. 1).

note that their diet contained small amounts of choline. This lack of agreement suggested that other members of the B-complex might have played a role, and for that reason the material was tried. All such experiments met with complete failure.

Discussion. Since the studies of Keye(8) and of Knaus(9) it has been rather generally accepted that an estrogen augments the motility of the uterus and progesterone opposes that action. Much of the work upon which this concept was based was done in rabbits, and it is entirely possible that the cat's uterus does not react in the same fashion. The studies of Knaus on the rabbit, confirmed by Reynolds and others, have made it clear that the uterus of this species responds differently from the cat to drugs and hormones. Bickers and Woods(10) among others, have emphasized the marked species differences in uterine physiology. Since progesterone enhances the responsiveness of the cat's uterus to drugs, this species would appear closer to the human in its physiologic reactions than the rabbit, since Kurzrok, Wiesbader, Mulinos, and Watson(11) reported that progesterone occasionally caused an increase in the tonus and amplitude of the contractions of the human uterus and could, if administered in the postmenstrual phase of the cycle, precipitate cramps. Wilson and Kurzrok(12) later reported that contractions were more marked, if less frequent, in the luteal phase of the cycle. They reported that posterior pituitary

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8. Keye, J. D., *Bull Johns Hopkins Hosp.*, 1923, v34, 60.

9. Knaus, H. H., *Arch. Gynak.*, 1930, v141, 374.

10. Bickers, W., and Woods, M., *Am. J. Obst. and Gynec.*, 1949, v58, 1099.

11. Kurzrok, R., Wiesbader, H., Mulinos, M. G., and Watson, B. P., *Endocrinology*, 1937, v21, 335.

12. Wilson, L., and Kurzrok, R., *Endocrinology*, 1938, v23, 79.

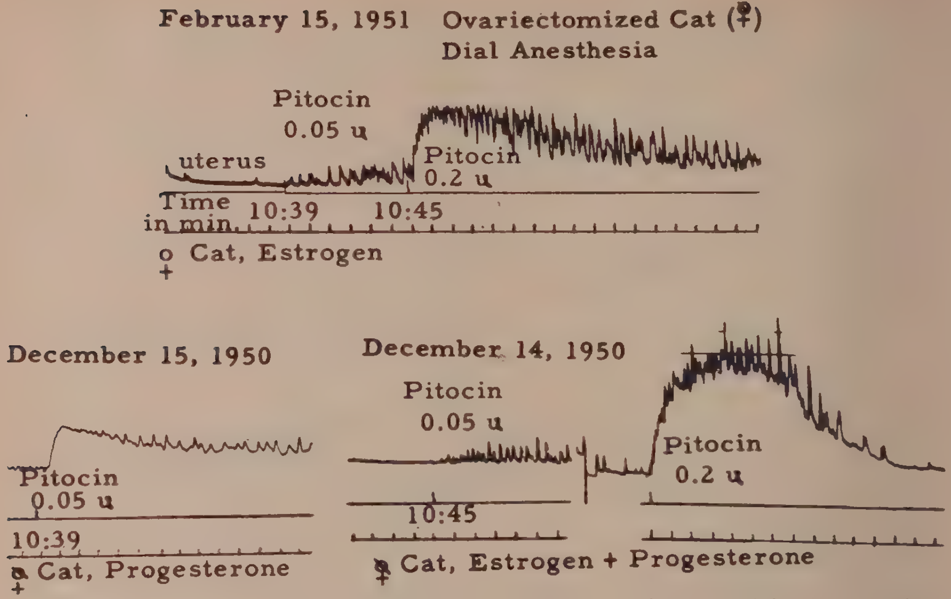

FIG. 3. *Feb. 15.* 1 mg α-estradiol dipropionate (intramusc) on first and third days. Experiment on ninth day. Pitocin units in total dose. *Dec. 15.* 5 mg progesterone (intramusc) on 4 consecutive days. Experiment on fifth day. *Dec. 14.* 0.3 mg α-estradiol dipropionate (intramusc) first through ninth day and 5 mg progesterone (intramusc) on days 8 and 9. Experiment on tenth day.

preparations gave reactions in all phases of the cycle and that the contractions of greatest amplitude were in the luteal phase. Others confirmed and extended this work(13,14) indicating that the effects of estrogens and progesterone on the human uterus must be viewed more as complementary than antagonistic. Recently Henry, Browne, and Venning(15) have entirely discarded the idea of an antagonistic action between estrogens and progesterone on the human uterus and presented evidence that the action must rather be synergistic. The present work would definitely support this concept in respect to the cat. Henry, Browne, and Venning(15) have stated that the maximal muscular efficiency of the uterus "requires adequate amounts of the two hormones in suitable proportions, and acting for a sufficient length of time." The present experiments are in accord with their views. For satisfactory results the hormonal preparation of the animals had to be initiated at least a week before the proposed experiment. The need for adequate proportions is emphasized by two facts, that pregnant cats, demonstrably with a good supply of gestational hormone, sometimes required supplementary estrogen to be responsive, and secondly that some normal, mature animals given only estrogen were not responsive, presumably due to a lack of endogenous progesterone. This suggests that above certain minimal amounts it is perhaps the ratio of the quantities of the two hormones present that determines the reactivity of the uterus to drugs.

Summary. A method has been described of treating cats with an estrogen plus progesterone to assure uteri *in vivo* highly responsive for hours to oxytocics. Modifications of this method applied to juvenile and castrated cats indicated that either type of

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13. Szarka, A., *Gynaecologia,* 1946, v122, 338 (Abstracted in *J. Obst. and Gynec., of British Empire,* 1947, v54, 702).

14. Moir, C., *Tr. Edinburgh Obst. Soc.,* 1934, v54, 93.

15. Henry, J. S., Browne, J. S. L., and Venning, E. H., *Am. J. Obst. and Gynec.,* 1950, v60, 471.

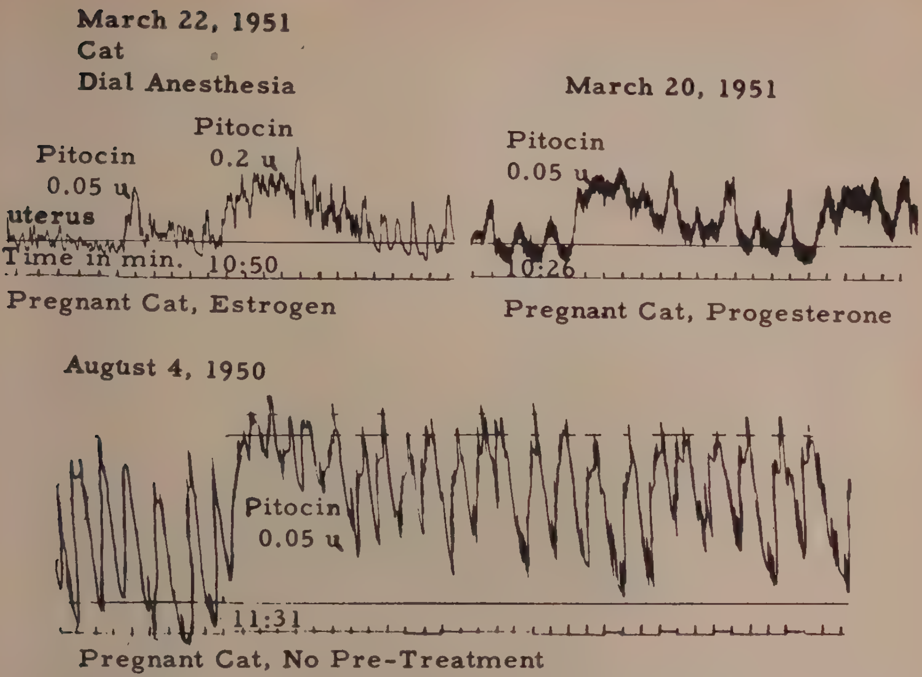

FIG. 4. *Mar. 22.* 1 mg α-estradiol dipropionate (intramusc) on first and third days. Experiment on sixth day. Units of pitocin in total dose. *Mar. 20.* 5 mg of progesterone (intramusc) on 4 consecutive days. Experiment on fifth day. *Aug. 4.* Cat in late stage of pregnancy. Responses of this nature were about as common as those demonstrating a complete lack of reactivity.

hormone would establish some brief uterine responsiveness but that both together were required to give a maximal and prolonged sensitivity to drugs. The two hormones in the cat are synergistic, or at least mutually complementary, and not antagonistic in respect to their effects upon the sensitivity of the cat's uterus *in vivo* to drugs.

Received June 5, 1951. P.S.E.B.M., 1951, v77.

Paper Chromatography of Protein Mixtures and Blood Plasmas. (18926)

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A technic for the 2-dimensional chromatography of blood plasmas of protein mixtures on filter paper has been described by Franklin and Quastel(1). It was found that the addition of surface active agents such as the "Tweens" or "Spans" appeared to facilitate separations of the protein constituents and extend the protein "pattern." The technic adopted at present is to add "Tween 85" or "Tween 81" to the plasma and use hemin as the protein marker. A mixture of the benzidine reagent and hydrogen peroxide is used to detect the hemin after employing $M/10$ sucrose solution as developing agent in the

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1. Franklin, A. E., and Quastel, J. H., Proc. Soc. Exp. Biol. and Med., 1950, v74, 803.

first dimension and $M/10$ sodium potassium tartrate solution in the second dimension. This technic has been used in the study of blood plasma composition after heparin administration and diets of fat meals(2), and in investigations of blood plasmas in multiple sclerosis(3). It has also been used in an investigation of the combination between thyroxine and plasma proteins(4).

It is the purpose of this paper to give further details concerning the movements of proteins on filter paper using the above technic, to show how certain proteins may be separated from each other on filter paper, to discuss the nature of the protein pattern and the effects of surface active agents and to present data concerning protein patterns found in various disorders.

Experimental procedure. Details of procedure are given in an earlier paper(1). It is important to note that with blood plasmas or protein mixtures not more than 0.02 ml aliquots should be applied to the paper, and with protein mixtures, aliquots should contain not more than 1 mg total protein. When larger quantities of protein are applied to the paper, protein separations are swamped by the effects of factors operating when a relatively large mass of protein is being slowly irrigated by the solutions used in this work. It is essential that there should be no overloading of the paper with the substances to be separated. When quantities of proteins, under the limits stated, are used, there is generally movement of the entire protein mass from the point of origin to the top of the paper within the experimental period of 1½ hours at room temperature. If a surface active agent is added to the protein mixture, however, the movement of certain proteins is greatly delayed and these may not all leave the point of origin in the first dimension.

Whatman No. 1 filter paper has been used almost exclusively. A variety of other papers has been tried; some papers (*e.g.* Schleicher and Schuell, No. 477 and 507) are quite unsuitable, while others (*e.g.* Whatman Nos. 2, 3, 3MM, 4) give results very similar to those obtained with Whatman No. 1. It is, therefore, important to adhere to one type of paper for the comparison of the protein patterns of plasmas from pathological cases with those of the normal.

It has been found that a convenient size of filter paper is an 8 in. square. Larger papers may give more extended patterns, but do not seem to offer any definite advantages.

Solutions of sucrose and sodium potassium tartrate have been selected after many experiments as the most suitable developing solvents in the first and second dimensions respectively. The former appears to facilitate movement of certain protein-hemin complexes, as well as uncombined hemin, in the first dimension; the latter allows movement of certain protein complexes in the second dimension whilst preventing movement of uncombined hemin. It has been found that in the presence of surface active agents (such as Tween 85) a very slight movement of hemin, when no proteins are present, may appear in the second dimension, but this is negligible compared with the movement of many protein-hemin complexes.

Protein chromatography of cytochrome c *solution.* When a solution of cytochrome *c** is applied to filter paper it is found that the substance moves either feebly or not at all in the first dimension using $M/10$ sucrose solution as developing solution, but is taken into the second dimension by $M/10$ sodium potassium tartrate solution. It is, of course, unnecessary to add hemin as protein marker to a solution of cytochrome *c* as this substance is itself detected with the benzidine reagent. The amount of cytochrome *c* moving in the second dimension is proportional to the amount placed at the origin. Photographs of chromatograms (no surface active agents being present) of various quantities of cytochrome *c* are shown in Fig. 1. The pigment, developed with the benzidine reagent,

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2. Swank, R. L., Franklin, A. E., and Quastel, J. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 850.

3. Swank, R. L., Franklin, A. E., and Quastel, J. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v76, 183.

4. Gross, J., Leblond, C. P., Franklin, A. E., and Quastel, J. H., *Science,* 1950, v111, 605.

* Kindly presented to us by Wyeth, Inc., Philadelphia, Pa.

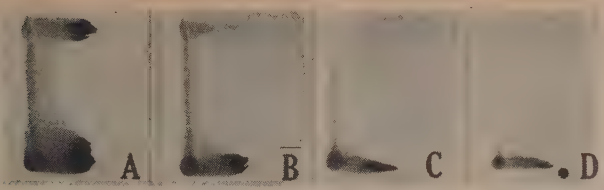

FIG. 1. Cytochrome *c* dilution series: A 133.3 γ, B 66.6 γ, C 33.3 γ, D 12.5 γ.

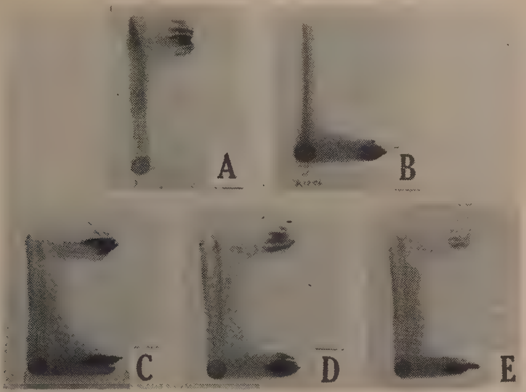

FIG. 2. Albumin and cytochrome *c* separations: A, 200 γ of crystalline human serum albumin (Cohn Fraction V); B, 40 γ of cytochrome *c*; C, Mixture of 200 γ of albumin and 80 γ of cytochrome *c*; D, mixture of 200 γ of albumin and 60 γ of cytochrome *c*; E, mixture of 200 γ of albumin and 40 γ of cytochrome *c*.

may be eluted from the spot in the second dimension with dilute HCl, yielding a yellow solution. The intensity of this color is measured with an electrophotometer. When high concentrations of cytochrome *c* are chromatographed, it is noted that a fraction appears at the top of the paper. This may be an impurity(5).

Paper chromatography of human serum albumin-cytochrome c *mixtures.* When mixtures of a solution of crystalline human serum albumin and a solution of cytochrome *c* are chromatographed on paper, a sharp separation of the two components appears to take place. There is sufficient hemin present in the cytochrome preparation (see also Holmes and Wynne(5) for evidence of breakdown of cytochrome *c* solution on standing) to form a complex with the albumin and serve as protein marker. Therefore hemin need not be added to the mixture before chromatography. The albumin-hemin complex moves with the advancing front in the first dimension, but the cytochrome is left behind. Both proteins move in the second dimension, albumin at the top of the paper and cytochrome at the bottom. Typical chromatograms of serum albumin-cytochrome *c* mixtures are shown in Fig. 2. It should also be noted that the intensity of color of the albumin fraction decreases with the concentration of cytochrome present, this being due to diminution of the amount of free hemin present.

Paper chromatography of protein mixtures. Preliminary investigations have shown that hemoglobin and cytochrome *c* preparations may also be separated on filter paper. McKerns(6) has reported the separation of the active component of rennin from both pure and crude preparations by the use of our technic, and has also followed changes undergone by casein during hydrolysis by rennin employing the same method.

Paper chromatography of plasma constitu-

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5. Holmes, W. L., and Wynne, A. M., *Fed. Proc., Am. Soc. Biol. Chem.*, 1951, v10, 199.

6. McKerns, K. W., *Canad. J. Med. Sc.*, 1951, v29, 59.

ents. (A) *Human serum albumin* (Cohn Fraction V). When a small quantity of a solution of a crystalline human (or bovine) serum albumin (Cohn Fraction V) is chromatographed, using sucrose and tartrate as developing solutions, it is found that the protein ascends in the first dimension with the advancing front as a large spot or a streak. The fact that the protein may ascend as a streak in the first dimension is probably due, largely, to gradual solution of the protein in the spot at the origin. In the second dimension, the streak is split by application of the second developing solution into a number of fractions yielding a "pattern." The extent of fractionation may depend on the length of the streak in the first dimension as well as upon the constituents of the developing solution. The various fractions seen in the second dimension do not necessarily represent different protein species; they may be composed of different molecular aggregations of the same protein, or differently ionized components of the protein, etc. It is, however, of importance that the fractionation observed may be duplicated with considerable regularity, and that the "pattern" observed with one protein differs significantly from that observed with another. A typical chromatogram of human serum albumin is shown in Fig. 3A.

The addition of a surface active agent to a solution of human serum albumin (*e.g.* 0.02 ml Tween 81 to 0.5 ml 6.5% albumin solution) results in a considerable lengthening of the protein spot in the first dimension. Application of the solvent in the second dimension splits the elongated spot into numerous fractions, giving rise to a pattern as shown in Fig. 3B and 3C.

It is known from the work of Putnam and Neurath(7) that albumins combine with surface active agents such as the detergents, to form complexes of various mole ratios. It is, therefore, likely that the elongation of the protein spot in the first dimension is partly due to the different rates of solution of the surface active agent-protein complexes, and

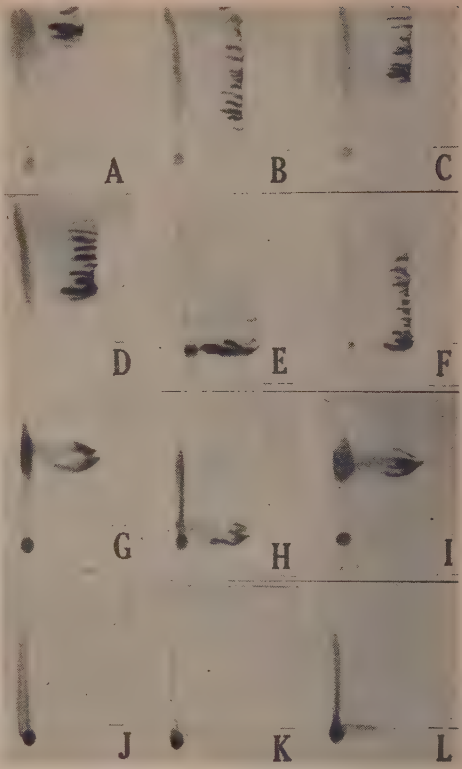

FIG. 3. Influence of Tween 81 and Tween 85 on the chromatograms of various plasma fractions. Cohn Fraction V (65 mg per ml) 95% albumin and 4% α-globulin: A, with 0.02 ml of 0.3% hemin added per 0.5 ml of solution; B, with 0.02 ml of 0.3% hemin and 0.02 ml of Tween 81 added per 0.5 ml of solution; C, with 0.02 ml of 0.3% hemin and 0.01 ml of Tween 85 added per 0.5 ml of solution.

Cohn Fraction IV—4 (65 mg per ml) 16% albumin, 46% α-globulin and 38% β-globulin: D, with 0.02 ml of 0.3% hemin added per 0.5 ml of solution; E, with 0.02 ml of 0.3% hemin and 0.02 ml of Tween 81 added per 0.5 ml of solution; F, with 0.02 ml of 0.3% hemin and 0.01 ml of Tween 85 added per 0.5 ml of solution.

Cohn Fraction II (65 mg per ml) 3% β-globulin and 97% γ-globulin: G, with 0.02 ml of 0.3% hemin added per 0.5 ml of solution; H, with 0.02 ml of 0.3% hemin and 0.02 ml of Tween 81 added per 0.5 ml of solution; I, with 0.02 ml of 0.3% hemin and 0.01 ml of Tween 85 added per 0.5 ml of solution.

Cohn Fraction I (65 mg per ml) 7% albumin, 8% α-globulin, 15% β-globulin, 9% γ-globulin and 61% fibrinogen: J, with 0.02 ml of 0.3% hemin added per 0.5 ml of solution; K, with 0.02 ml of 0.3% hemin and 0.02 ml of Tween 81 added per 0.5 ml of solution; L, with 0.02 ml of 0.3% hemin and 0.01 ml of Tween 85 added per 0.5 ml of solution.

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7. Putnam, F. W., and Neurath, H., *J. Amer. Chem. Soc.*, 1944, v66, 1992.

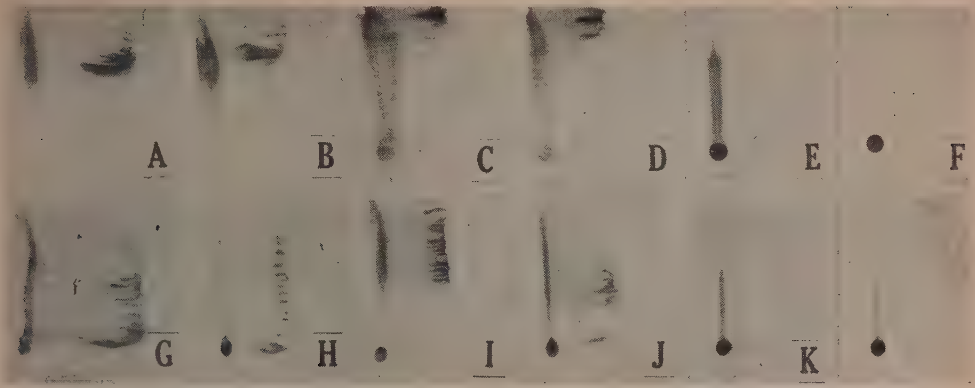

FIG. 4. Influence of Tween 81 on the chromatograms of various plasma fractions (0.02 ml of 0.3% hemin added per 0.5 ml of mixture throughout): A, human plasma; B, reconstituted plasma; C, 3% Cohn Fraction V; D, 1.3% Cohn Fraction IV-4; E, 0.5% Cohn Fraction II; F, 0.6% Cohn Fraction I; G, same as A with 0.02 ml of Tween 81 added per 0.5 ml; H, same as B with 0.02 ml of Tween 81 added per 0.5 ml; I, same as C with 0.02 ml of Tween 81 added per 0.5 ml; J, same as D with 0.02 ml of Tween 81 added per 0.5 ml; K, same as E with 0.02 ml of Tween 81 added per 0.5 ml; L, same as F with 0.02 ml of Tween 81 added per 0.5 ml.

partly due to the varying affinities of these complexes to the cellulose of the paper (see also ref. 8). The increase, therefore, in the number of fractions in the second dimension is probably largely due to the formation of these complexes.

(B) *α and β globulins* (Cohn Fraction IV-4). Paper chromatography of a solution of Cohn Fraction IV-4 containing a mixture of α and β globulins and some albumin shows much higher fractionation in the second dimension in the absence of a surface active agent, than is observed with a solution of the same quantity of serum albumin (see Fig. 3D). The addition of Tween 81 to a solution of these proteins results in a very greatly diminished rate of movement of the proteins in the first dimension, probably due to complex formation. Movement of the complexes takes place in the second dimension as shown in Fig. 3E.

Experiments have shown that the rate of ascent of globulin on the filter paper after addition of a surface active agent, is much more restricted in the first dimension than that of albumin. This may result, when using Tween 81, in little movement of the protein in the first dimension with a consequent heavy streak at the bottom of the paper in the second dimension (Fig. 3E). When a surface active agent such as Tween 85 is used, an elongated spot, commencing at the origin, appears in the first dimension resulting numerous fractions, in the second dimension (Fig. 3F). The inference has been that fractions due to albumin are in the upper half of the chromatogram; those due to globulins are in the lower half.

(C) *γ globulin and fibrinogen.* Movement of γ globulin in the absence of surface active agent takes place in the first dimension with but little movement in the second dimension. This may be contrasted with the ease of movement of the α and β globulins in the second dimension. The presence of a surface active agent, however, causes a reduced rate of movement of the protein in the first dimension, and movement at the bottom, or in the lower half, of the chromatogram in the second dimension. Fibrinogen moves but little in either first or second dimension, with or without surface active agents. Chromatograms illustrating these results are shown in Fig. 3G, H, I, J, K, and L.

Paper chromatography of plasma, and of reconstituted plasma. The chromatogram of a mixture of albumin, α, β, and γ globulins and fibrinogen at the concentrations present

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8. Hanes, C. S., and Isherwood, F. A., *Nature*, 1949, v164, 1107.

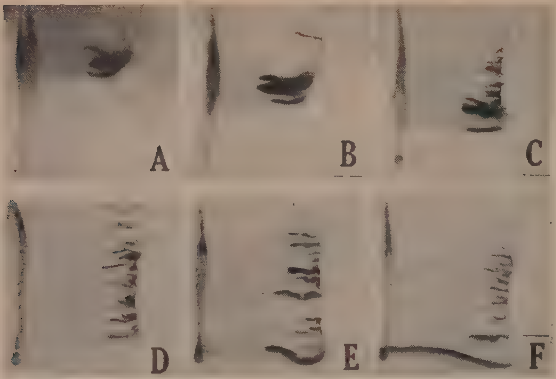

FIG. 5. Influence of Tween 85 on chromatograms of serum albumin, globulin and plasma mixtures: A, human plasma; B, human plasma + 30 mg Cohn Fraction V (95% human serum albumin) added per 0.5 ml of plasma; C, human plasma + 30 mg Cohn Fraction II (97% γ-globulin) added per 0.5 ml of plasma; D, same as A with 0.01 ml of Tween 85 added per 0.5 ml of plasma; E, same as B with 0.01 ml of Tween 85 added per 0.5 ml of plasma; F, same as C with 0.01 ml of Tween 85 added per 0.5 ml of plasma.

in plasma resembles very closely the chromatogram of plasma itself (Fig. 4). It is, therefore, apparent that the chromatogram of a complex mixture such as plasma may be regarded largely as a composite of the chromatograms of the individual proteins. This may not always be the case, for plasma may contain other constituents, such as surface active agents, which may alter the pattern(2) and which may not be present in the isolated protein fractions from plasma.

Effects of dilution of plasma. The addition of water to blood plasma brings about no apparent change in the chromatogram pattern, with or without surface active agents, until 50% dilution has been reached. Further dilution causes significant changes in the pattern, particularly when Tween 85 is used as surface active agent.

Effects of addition of serum albumin or of γ globulin to human plasma. Addition of 30 mg human serum albumin to 0.5 ml human plasma causes little obvious change in the chromatogram pattern (Fig. 5A, B, D, E). On the other hand the addition of 30 mg of γ globulin to 0.5 ml human plasma brings about a marked alteration of the pattern (Fig. 5C, F), a heavy fraction appearing from the point of origin in the second dimension, when Tween 85 is used. This heavy fraction at the bottom of the pattern is typical of the chromatograms of pathological cases in which the globulin/albumin ratio of the plasma has been increased.

Use of dyes as protein markers. We have found that proteins without the addition of hemin may be detected by treating the chromatogram with a dilute alcoholic solution of bromthymol blue, followed by a thorough washing with water. This procedure gives rise to a pattern identical with that obtained using hemin as protein marker, followed by application of the benzidine reagent.

Papastamatis and Wilkinson(9) have also shown that bromthymol blue may be effectively used for the marking of proteins on paper, using a variety of developing solutions. The method was used to detect many classes of proteins (*e.g.* insulin, plasma proteins, egg albumin) subjected to paper chromatography, 20 γ of protein being detectable.

Paper chromatography of blood plasmas of various disorders. It has been found that the paper chromatograms of normal blood plasmas yield patterns, which are surprisingly similar and which duplicate with considerable regularity. Normal patterns have already been published(1-3) and a typical example is shown in Fig. 6A, B.

The patterns exhibited in chromatograms of

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9. Papastamatis, S. C., and Wilkinson, J. F., *Nature,* 1951, v167, 724.

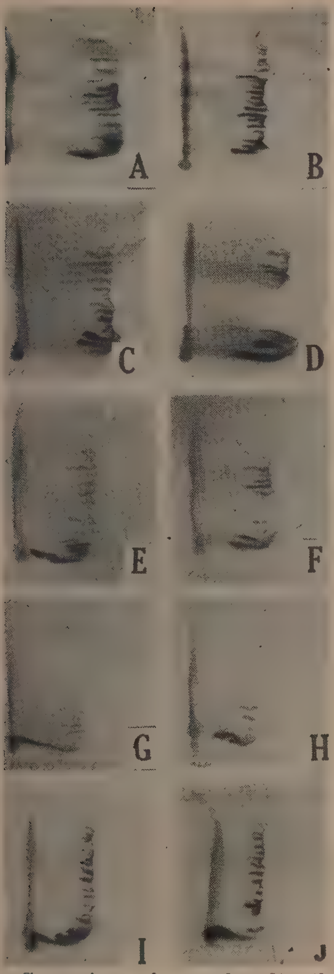

FIG. 6. Comparison of normal and pathological plasma chromatograms employing Tween 81 and 85: A, plasma of normal patient (+ 0.02 ml of Tween 81 added per 0.5 ml of plasma); B, plasma of same patient (+ 0.01 ml of Tween 85 added per 0.5 ml of plasma); C, plasma of a patient with multiple myeloma (+ 0.02 ml of Tween 81); D, plasma of same patient (+ 0.01 ml of Tween 85); E, plasma of a patient with liver cirrhosis (+ 0.02 ml of Tween 81); F, plasma of same patient (+ 0.01 ml of Tween 85); G, plasma of a patient with cancer of the stomach (+ 0.02 ml of Tween 81); H, plasma of same patient (+ 0.01 ml of Tween 85); I, plasma of a patient with lupus erythematosus (+ 0.02 ml of Tween 81); J, plasma of same patient (+ 0.01 ml of Tween 85).

pathological plasmas vary greatly, according to the disease, and usually show distinct changes from the normal.

It would be expected, from the results given above, that plasmas having different albumin-globulin ratios from the normal would exhibit patterns different from the normal. This in fact is the case. Plasmas, such as those from patients with multiple myeloma or liver cirrhosis, where there are large changes in the albumin-globulin ratio, yield chromatograms which are characterized by the absence, or relative weakness, of the upper fractions (due to albumin) and by the presence of heavy basal fractions (due to globulins). (Fig. 6C, D, E, F). Chromatograms are also shown of plasmas of patients suffering from cancer of the stomach and from lupus erythematosis (Fig. 6G, H, I, J,). Whether such chromatograms of plasmas of pathological cases will prove to be of value in diagnosis remains to be seen, but sufficient work has been done to show that the chromatograms duplicate with regularity in the same patient, so long as the clinical condition remains the same. They vary during the course of the disease and may revert to normal with successful medical treatment. More extensive results obtained with pathological cases will be published elsewhere.

Paper chromatography of snake venoms. It was of interest to chromatograph snake venoms of various types, to see if the patterns differ substantially from one another. Using 0.8 mg of dried venom, it has been found that the chromatograms of 2 cobra venoms are almost identical, those of 2 specimens of Russel viper venom are very similar to those of the cobra venom, whilst that of a *Agkistrodon piscivorus* has a pattern differing markedly from those of other snake venoms (Fig. 7). The venoms were chromatographed without the addition of surface active agents.

Summary. Further details are given concerning the movements of proteins on filter paper using $M/10$ solutions of sucrose solution and sodium potassium tartrate as developing solvents in the first and second dimension, respectively. It is shown that mixtures of proteins, such as serum albumin and cytochrome *c*, may be separated from each other on paper, since the former moves in the first dimension, whilst the latter does

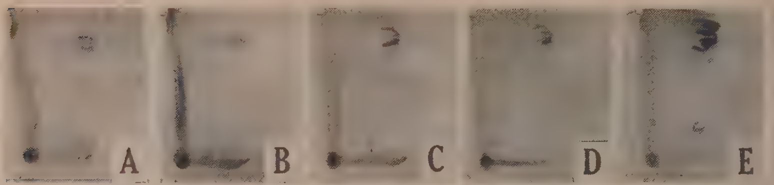

FIG 7. Snake venoms: A, cobra venom supplied by Haffkine Institute, Bombay, India; B, cobra venom supplied by Hynson, Westcott and Dunning, Inc., Baltimore, Md.; C, Russel viper venom supplied by Haffkine Institute, Bombay, India; D, Russel viper venom supplied by Wellcome Research Labs., Langley Court, Beckenham, England; E, *Agkistrodon piscivorus* supplied by Ross Allen's Reptile Institute, Silver Springs, Fla.

not and both proteins move in the second dimension. The amount of cytochrome *c* moving in the second dimension is proportional to the amount applied to the paper. The chromatographic patterns of purified proteins *e.g.* crystalline human serum albumin, α, β, and γ globulins, and fibrinogen differ from each other in a characteristic manner. The differences are more marked when the proteins are mixed with surface active agents with which they form complexes; and these complexes move at different rates on filter paper. Complexes of surface active agents with albumin move with greater ease along the first dimension than those formed with globulins; the latter tend to remain at the point of origin. Almost all the protein complexes, however, move in the second dimension. The pattern given with a blood plasma is a composite of the patterns of the individual protein fractions, the basal fractions being apparently due to globulin. The patterns of chromatograms of pathological cases (multiple myeloma, liver cirrhosis, cancer of the stomach, lupus erythematosis) differ significantly from the normal, and are often characterized by the presence of heavy basal fractions due probably to increase in the globulin content of the plasma.

The chromatograms of dried snake venoms (cobra, Russel viper, *Agkistrodon piscivorus*) show various distinctive features.

We are greatly indebted to the National Cancer Institute of Canada and to the Rockefeller Foundation for grants which have made this work possible and to the Cutter Laboratories, Berkeley, Cal. for their generous donation of purified plasma protein fractions.

We also wish to acknowledge the kind assistance of Drs. R. R. Fitzgerald, G. E. Joron and F. Kalz in providing us with blood samples from their patients.

Received June 27, 1951. P.S.E.B.M., 1951, v77.

Effect of Pretreatment with Cysteine on Survival of Mice Exposed to External and Internal Irradiation.* (18927)

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Patt, Tyree, Straube and Smith(1-3) reported that rats injected intravenously with cysteine, shortly before irradiation with lethal

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[*] Carried out under Contract AT-(40-1)-269 with the Division of Biology and Medicine, United States Atomic Energy Commission.

1. Patt, H. M., Tyree, E. B., Straube, R. L., and Smith, D. E., *Science*, 1949, v110, 213.

2. Patt, H. M., Smith, D. E., Tyree, E. B., and Straube, R. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 18.

3. Smith, D. E., Patt, H. M., Tyree, E. B., and Straube, R. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 198.

doses of x-rays, survive considerably longer than irradiated, but not pretreated controls. Cronkite, Chapman and Brecher(4-6) obtained similar results in Swiss mice prepared by subcutaneous injection of another sulfhydril compound glutathione. We attempted, in experiments reported below, to analyze the mechanism of cysteine effect on irradiated mice by varying several factors of the experiment: (1) source of radiation; (2) doses of radiation; (3) dose and route of administration of cysteine; (4) strain of mice; (5) absence or presence of serous cellular exudate in the peritoneal cavity.

Material and methods. 1. *Animals.* Mice of dba and CFW strains, of 25 g weight, were used. In one series they were inoculated intraperitoneally 4 days before irradiation with 1 million Sarcoma 37 cells from serial peritoneal transfers(7). Total and differential counts of cells in their peritoneal fluid (accumulating as result of inoculation) were carried out repeatedly in irradiated mice and controls. The technic of inoculation, of fluid withdrawal and of cell counts was described elsewhere(7). 2. *Cysteine.* 2.5 to 10% solution (pH 1.0) was prepared a few minutes before injection and in several experiments adjusted to pH 6.0 or 6.5 with 8% NaOH solution. Doses of 0.1 or 0.2 cc of these solutions were injected into mice, intravenously, intraperitoneally or subcutaneously, about 10 to 15 minutes before irradiation. In a few preliminary control experiments the cysteine was injected 20 minutes after irradiation. 3. *Irradiation.* Doses of 300 to 800 of filtered x-rays (Filter: 1.2 mm Sn; 0.25 mm Cu; 1.0 mm Al; TSD 50 cm; HVL 3.27 mm Cu; 250 PKV) were used for external irradiation and doses of 0.8 mc of radioactive colloidal gold† for internal irradiation (intraperitoneal injections). For technical reasons, groups of not more than 10 mice were irradiated in each experiment and the results were pooled. 4. *Survival.* The total period of survival varied in different experiments, but for the period between 10 and 14 days the results were consistent in all experiments and therefore they could be pooled for recording.

Results. The preliminary experiments on after-treatment of irradiated mice with cysteine confirmed the observation of other authors(1-3) that this procedure does not influence survival of mice.

For easier reading and understanding the experiments reviewed in Table I are divided into 4 series illustrating (A) longer survival of x-irradiated mice pretreated with small doses of cysteine; (B) rapid death after irradiation, with x-rays of mice pretreated with high doses of cysteine; (C) failure of the procedure used in B in mice possessing large amount of peritoneal fluid; (D) lack of effect of pretreatment with cysteine in mice irradiated with radioactive gold.

Repeated examinations of peritoneal fluid in sarcoma inoculated mice were carried out in order to study the influence of x-ray treatment, with or without cysteine pretreatment on the following characteristics of the fluid: 1. Total number of cells (sarcoma cells and leukocytes) per cmm of fluid at various intervals after irradiation (Fig. 1). 2. Approximate volume of fluid in the peritoneal cavity. It was found that after x-ray treatment, with or without cysteine, the volume of peritoneal fluid increased in most mice of the series C 4 to 8 times within 48 hours. Differential counts showed that later increase in cell number in cysteine-x-ray group was paralleled by a rise in the percentage of lymphocytes and polymorphonuclears. Thus, there was at that stage, an absolute increase in cell number in the fluid and not only a relative increase due to partial loss of fluid. On the contrary in the peritoneal fluid of animals treated *i.p.* with radioactive gold, with or without cysteine, the predominant feature was the increase in cell concentration by progressive disappearance of fluid which became very scarce within 5 days after treatment.

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5. Cronkite, E. P., Brecher, G., and Chapman, W. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 396.

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† Gold was obtained through Abbott Research Laboratories, Dr. D. L. Tabern, North Chicago, Ill.

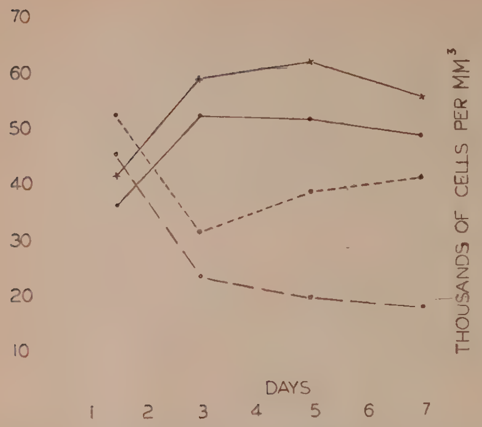

FIG. 1. Each graph represents average of 20 mice. x————x No treatment; more than 30% sarcoma cells. •————• Cysteine alone; more than 30% sarcoma cells. •– – – – – – –• X-rays alone; less than 15% sarcoma cells. ° — — — ° Cysteine and X-rays; less than 15% sarcoma cells.

Tumor cells disappeared from the peritoneal fluid of gold treated animals, as it was expected(8). In all x-ray treated mice, there was a destruction of majority of tumor cells (as shown by comparison with untreated controls) and in some cases of all tumor cells, as demonstrated by negative results of implantation of large amounts (0.25 cc) of peritoneal fluid into new mice.

Discussion. The data of the series A experiments demonstrate that small doses (125 to 250 mg/kg weight) of cysteine injected before irradiation increased, at least temporarily, the survival of mice exposed to a lethal dose of x-rays. The importance of species, strain and several environmental or experimental factors in this type of experiments is demonstrated by: 1. great variations in survival of mice after the first two weeks following irradiation; 2. higher percentage of survivors, under the same experimental conditions, in dba mice than in CFW mice; 3. better protection obtained by similar technic in rats by Patt *et al.*(1-3). Decrease of cysteine (pH 1.0) activity after alkalinization to pH 6.0-6.5 (Table I) or to pH 7.0-7.5(1) was due evidently to partial denaturation of this amino acid by oxidation.

In new mice pretreated intraperitoneally with higher doses (more than 800 mg/kg) of cysteine (Series B, Table I), the irradiation even with lower (300 r) doses of x-rays provoked instantaneous death or agony lasting a few hours. The higher activity of cysteine at pH 1.0 was not due to its acidity, as shown by control experiments with saline solution at pH 1.0. It is obvious that either the biological effect of x-rays is potentiated by high doses of cysteine, or vice versa, the x-ray treatment enables blood cysteine to gain access promptly to some highly vulnerable body tissues. The first alternative is contradicted by well known facts that decrease of oxygen content in tissue by a reducing agent, such as cysteine, may attenuate but not enhance the effect of x-ray. There is more evidence in favor of the second alternative. Several authors(9-12) reported increased permeability of vessels and exudation following closely the irradiation. While in normal animals exposed to lethal dose of x-rays, there is a latent period of 1-2 hours before development of clinical symptoms(13), there is evidence(14) that functional changes (vasodilatation, exudation) and even histological changes(9) occur already during irradiation. In our experiments (Series C) in animals possessing peritoneal exudate, an increase in the amount of this fluid was noticeable within 2 hours after irradiation. The resistance of mice possessing copious peritoneal exudate to immediate lethal effect of high doses of cysteine (Series C) indicated that the exudate possesses some factor rapidly inactivating (oxidizing) cysteine. This conclusion was confirmed by the observation that

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TABLE I. Effect of Pretreatment with Various Doses of Cysteine on Survival of Mice, New or Inoculated i.p. with Sarcoma Cells, After Irradiation with X-rays or Radioactive Gold.

Mice		Pretreatment with cysteine			Irradiation		% of surviving mice	
Strain	No.	pH of solution	Dose, mg	Route	Source	Dose	Immediately after irradiation	After 10 to 14 days
Series A CFW	50	1	2.5	i.p. i.v. s.c.	filtered x-rays	600r	100	75 65 50
	50	6	5	i.p. i.v. s.c.	"	600r	100	85 70 60
	25	Controls not pretreated			"	600r	100	40
	30	1	2.5	i.p. i.v. s.c.	Controls not irradiated		100	100 100 100
dba	50	1	2.5	i.p. i.v. s.c.	filtered x-rays	600r	100	85 75 60
	50	6	5	i.p. i.v. s.c.	"	600r	100	90 75 70
	25	Controls not pretreated			"	600r	100	50
Series B CFW	50	1	20	i.p. i.v.	"	300 to 600r	0 50	0 0
	30	Untreated controls			"	300 to 600r	100	45
	50	1	20	i.p. i.v.	Controls not irradiated		100	50
	30	Controls saline solution pH 1		i.p. i.v.	filtered x-rays	300 to 600r	100	80 45
	20	6	40	i.p. i.v.	"	600r	60 70	60 70
	20	6	40	i.p. i.v.	Not irradiated controls		100	100
Series C CFW mice inoculated with sarcoma	50	1	20 or 40	i.v. i.p.	filtered x-rays	300 to 600r	100 100	40 35
Series D CFW	30	1	2.5	i.v.	radioactive colloidal gold	0.8 mc. i.p.	100	10
	30	6	5	i.v.	"	0.8 mc. i.p.	100	20
	20	1	2.5	i.v.	"	0.8 mc. i.p.	100	20
	20	6	5	i.v.	"	0.8 mc. i.p.	100	10
	30	Controls not pretreated			"	0.8 mc. i.p.	100	25

doses of 20 mg of cysteine mixed *in vitro* with 0.25 cc of peritoneal fluid and immediately injected into new mice were well tolerated. Presuming that the fluid transuded from the vessels by x-ray treatments possesses the ability of peritoneal exudate to inactivate cysteine, the lack of protective action of aftertreatment with cysteine may be interpreted as inactivation of cysteine by the fluid transuded from irradiated vessels before cysteine injection.

Studies of peritoneal exudate in animals inoculated *i.p.* with sarcoma suggested an interpretation for "protective action" of small doses of cysteine, as compared with "potentiating action" of high doses. It was found that the total number of lymphocytes and polymorphonuclears decreases steadily after treatment with x-rays alone, but in mice pretreated with cysteine this depression of leukocytes was only temporary and followed by increase in cell number. This increase was lacking in mice pretreated with high doses of cysteine. These results suggest that small doses of cysteine stimulate and high doses depress some cellular metabolic processes, perhaps, the activity of hematopoietic tissue as suggested by Cronkite(5,6). Since intraperitoneal route was most efficient for protection of mice by small doses of cysteine and for inducing their death by high doses, it may be presumed that some abdominal organs are the site of action of cysteine.

It follows from the experiments of the Series D that pretreatment with small doses of cysteine acted in internally (intraperitoneally) irradiated mice in a way similar to the effect of high doses in externally irradiated mice *i.e.*, it reduced their life span. It is reasonable to presume that this phenomenon is effected by a similar mechanism, *i.e.*, the increased vascular permeability of irradiated tissues enables cysteine to reach some vitally important tissues faster and in higher concentration.

Conclusions and summary. 1. CFW and dba mice were pretreated with small (125 and 250 mg/kg) doses of cysteine (pH 1.0 or 6.5) before irradiation with lethal doses of x-rays (600 r). It was found that 10 to 14 days after irradiation the percentage of surviving animals was consistently higher in cysteine pretreated group than in irradiated not pretreated controls. These results illustrate "protective" effect of cysteine against radiation. 2. In new mice prepared with high doses (more than 800 mg/kg) of cysteine the x-ray treatment was followed immediately by death or agony. In mice treated by one of these factors alone, the condition was affected only after several days. Thus, the rapid lethal effect of combined treatment with cysteine (high doses) and x-rays is interpreted as potentiation of one factor by the other. 3. This effect of high doses of cysteine was absent in x-ray treated mice possessing copious peritoneal exudate due to the growth of free S-37 cells in the peritoneal fluid. This failure of cysteine is attributed to inactivation (oxidation) of cysteine by some factor of the peritoneal fluid. In these mice with exudate pretreated with small doses of cysteine, the decrease in the cell number (in the exudate) induced by irradiation was followed by a steady increase, while in irradiated controls the decrease continued until their death. 4. Pretreatment with small doses of cysteine (intravenously) had unfavorable effect on survival of mice injected (*i.p.*) with radioactive colloidal gold. 5. An explanation of the above phenomenon as manifestation of cysteine penetration from blood stream into tissues after increase of blood vessels permeability by high doses of irradiation is offered.

Received June 27, 1951. P.S.E.B.M., 1951, v77.

Use of N-Acetyl 4-Aminoantipyrine (NAAP)* in Measurement of Total Body Water. (18928)

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Previous studies have demonstrated that antipyrine is a satisfactory substance for use in the estimation of total body water(1). Antipyrine, however, does not entirely fulfill the requisites of an ideal agent to use for the measurement of body water. It is bound to plasma proteins to the extent of about 10%. Compensatory binding in tissues, presumably, accounts for the fact that the substance diffuses throughout the body in close proportion to the water content. Again, a correction must be applied for the antipyrine metabolized during the period of measurement. This involves the analysis of three consecutive plasma levels.

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* N-acetyl 4-aminoantipyrine may be purchased from the special chemicals division, Winthrop-Stearns, Inc., 1450 Broadway, New York City.

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In the study of the intermediary metabolism of aminopyrine (4-dimethylaminoantipyrine) a metabolite, N-acetyl 4-aminoantipyrine (NAAP), was found which also distributes in tissues in proportion to their water contents (2). In contrast to antipyrine, NAAP is negligibly bound to plasma proteins. Since its rate of metabolism is essentially nil, its volume of distribution may be calculated from a single plasma sample and a urine sample. Furthermore, the determination of NAAP does not require an ultraviolet spectrophotometer. For these reasons, it is believed that NAAP has certain advantages over antipyrine in the estimation of total body water.

Chemical methods. NAAP in organ tissues is estimated by a procedure previously described(2). NAAP in plasma is assayed in the filtrate after zinc hydroxide deproteinization. The urine (generally diluted 1 to 50) is handled similarly. NAAP is hydrolyzed in acid and the resulting 4-aminoantipyrine is estimated by diazotization and coupling with alpha naphthol.

Estimation of NAAP in plasma and urine. To 2 ml of plasma or diluted urine (containing 20 to 60 μg of NAAP) in a 50 ml flask, add 3 ml of water and 2 ml of zinc reagent (100 g of $ZnSO_4 \cdot 7H_2O$ and 40 ml of 6N sulfuric acid, diluted with water to 1 liter). Add 2 ml of 0.75N NaOH dropwise with continuous swirling of the flask and shake for a half minute. After 10 minutes, transfer to a test tube, and centrifuge. Transfer 4 ml of the clear supernatant fluid to a colorimeter tube and add 1 ml of 2.5 N HCl. Cover the tube with a glass marble to prevent evaporation and heat in a boiling water bath for 30 minutes. Cool and add 0.5 ml of 0.2% sodium nitrite solution and place in refrigerator for 10 minutes. Then add 0.5 ml of 1% ammonium sulfamate solution and mix. Wait 3 minutes and add 0.1 ml of a 5% solution of resublimed alpha naphthol in absolute alcohol, followed by 1 ml of 4N NaOH. Let stand for about 5 minutes and read at 490 mμ in a photoelectric colorimeter. A reagent blank run through the procedure is used for the zero setting.

Standards are prepared by taking 4 ml of a known solution of NAAP (5 to 10 μg per ml), adding acid, and hydrolyzing and colorizing as with the unknowns. Standards should be run concurrently with the unknowns since there are slight daily variations in color intensity.

NAAP added to plasma in amounts from 15 to 30 μg per ml has been estimated with an average recovery of 101.5% with a standard deviation of 2.4% (112 recoveries).

Results. Distribution of NAAP in body tissues. Two dogs were given NAAP intravenously. Four hours later, they were killed by an intravenous injection of air. The tissue concentration of NAAP was measured and calculated in terms of tissue water. Tissue water was determined by drying at 95-100°. The concentration of NAAP in the water of the tissues was almost identical with that of plasma water (Table I). These results indicate that NAAP achieves a uniform concentration in all the fluid compartments of the body.

The extent to which NAAP is bound to nondiffusible constituents of plasma was determined by dialysis against isotonic phosphate of pH 7.4 at 37° for 20 hours. The results indicated binding of less than 3%.

Urinary excretion of NAAP. One gram of NAAP was administered intravenously to each of 6 normal human subjects. Forty-eight hour urine collections showed a recovery of NAAP which averaged 92% (range 82 to 100%). Thus the disappearance of NAAP from the body can be almost entirely accounted for by its renal excretion and only a minor fraction of the compound is metabolized over a period of 3 hours (0-15 mg of a 1 g dose).

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TABLE I. Distribution of N-Acetyl 4-Aminoantipyrine (NAAP) in Water of Dog Tissues. Dog 1, weighing 7.3 kg, received 2.1 g and dog 2, weighing 13.7 kg, received 2.5 g of NAAP intravenously. The tissues were examined 4 hr later.

Tissue	NAAP in tissue water, mg per l		Ratio, tissue water NAAP/plasma water NAAP	
	Dog 1	Dog 2	Dog 1	Dog 2
Plasma	317	142		
Red cells	310	141	.98	1.00
Liver	340	139	1.07	.98
Spleen	308	133	.97	.94
Muscle (leg)	334	144	1.05	1.01
Muscle (back)	315	144	.99	1.01
Lung	316	152	1.00	1.07
Heart	308	158	.97	1.10
Brain	268	137	.85	.97
Cerebrospinal fluid	278	123	.89	.87
Avg			.97	.99

TABLE II. Distribution of N-Acetyl 4-Aminoantipyrine (NAAP) Between Plasma and Transudates. Subjects with peripheral edema, ascites, and pleural effusion were given 1 g of NAAP intravenously and the plasma and transudate were obtained 5½ to 8 hr subsequently.

Subject No.	Time after NAAP administration, hr	NAAP, mg per l water	Ratio, transudate/plasma water
1. Edema fluid	5½	18.9	.80
Plasma		23.5	
Edema fluid	6½	20.1	.89
Plasma		22.6	
Edema fluid	8	22	.97
Plasma		22.6	
2. Pleural fluid	6½	15	.73
Plasma		20.6	
3. Pleural fluid	6½	13.6	.68
Plasma		20	
4. Pleural fluid	8	7.1	.49
Plasma		14.7	
5. Ascitic fluid	7	16.6	.79
Plasma		21.1	

Time for even distribution of NAAP in body. One gram of NAAP was administered intravenously to each of 20 individuals in a total of 30 experiments. The logarithm of the plasma concentrations plotted against time (3, 4½ and 6 hours) yielded without exception a linear relationship. The rate of disappearance from plasma averaged 8% per hour with only minor variation. It may be inferred that equilibration of NAAP with body water is complete within 3 hours for normal subjects.

One gram of NAAP was administered to 5 subjects with abnormal accumulations of fluid. The concentration of NAAP in plasma water and the fluid accumulation was compared 5½ to 8 hours later (Table II). The concentration of NAAP in the transudate was usually considerably less than in plasma water, indicating incomplete distribution of the compound. To 2 of the subjects (1 and 2), 1 g of antipyrine was administered concurrently with the NAAP. In contrast to NAAP, the concentrations of antipyrine in the 2 fluids were almost identical in 5½ hours. These results suggest that in subjects with abnormal fluid depots, NAAP equilibrates too slowly between plasma and fluid accumulations to use it for estimation of total fluid.

Toxicity. No toxic effects have been observed in 30 subjects given 1 g of NAAP.

Measurement of body water with NAAP. Extrapolation procedure. A control sample of blood is drawn for the estimation of a small blank value which is used for correcting subsequent samples. One gram of NAAP in 50 ml of 5% glucose solution is injected intravenously from a burette during a period of

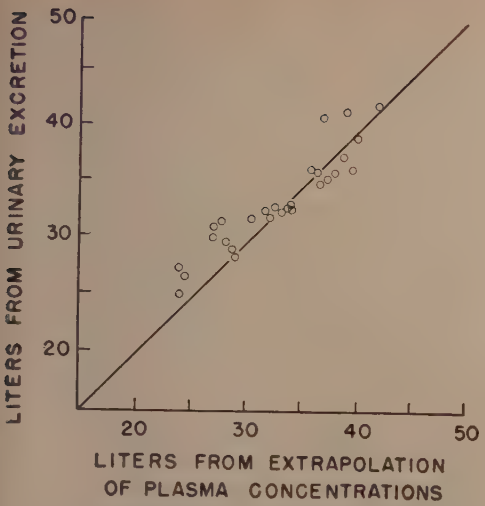

FIG. 1. Comparison of extrapolation and urine collection procedures for measuring total body water with N-acetyl 4-aminoantipyrine.

about 5 minutes. The middle of the injection period is taken as zero time. Blood samples are subsequently withdrawn at about 3, 4½ and 6 hours and the exact time noted. All blood samples are heparinized. Plasma and cells are separated by centrifugation and the plasma stored in stoppered tubes for subsequent analysis. NAAP is stable in plasma for at least several days at refrigerator temperature.

Body water is calculated from the plasma levels in the manner previously described for the antipyrine method(1).

Urine collection procedure. Since NAAP is almost quantitatively eliminated in the urine of humans, it should be possible to calculate its volume of distribution on the basis of its urinary excretion and a single plasma level. This involves the measurement of a plasma level and the urinary excretion 3 hours following the administration of NAAP. Body water is calculated as follows:

$$\text{Body water (liters)} = \frac{\text{amt of drug injected (mg)} - \text{amt excreted (mg)}}{\text{conc. in plasma water (mg per liter)}}$$

The procedure possesses two inherent sources of error: the small amount of NAAP metabolized in the body (0 to 15 mg of a 1 g dose in 3 hours) and the NAAP that is concentrated in the urine left in the ureter and pelvis after the bladder is completely emptied. These errors are in the direction of making the estimate of body water too large. The magnitude of the combined errors was assessed by comparing the volume of dilution of NAAP as measured from a single plasma level and urinary excretion, with that calculated by extrapolation of a line through 3 plasma concentrations. Comparisons for 30 subjects were in fairly good agreement; those obtained by the urine collection procedure averaging 1 liter higher than those obtained by the plasma extrapolation technic (Fig. 1). These results suggest that the errors of the urine collection procedure are not large.

Comparison of body water measurements made with antipyrine and NAAP. The volume of distribution of NAAP (extrapolation tech-

TABLE III. Comparison of Total Body Water in Normal Subjects Measured by N-Acetyl 4-Aminoantipyrine (NAAP) and Antipyrine.

Subject No.	Wt, kg	Total water: NAAP, liters	Total water: Antipyrine, liters	Difference (NAAP–antipyrine), liters
1	43.7	24	22.3	+1.7
2	47.8	27.3	28.8	—1.5
3	53.7	27	30.5	—3.5
4	56.5	27	29.6	—2.6
5	58.7	33	32.5	+ .5
6	60.4	33.8	37	—3.2
7	62	29.5	30.1	— .6
8	62.5	36.5	36.4	+ .1
9	68.6	28.2	30.4	—2.2
10	72.3	40	43.3	—3.3
11	77.3	33.4	35.8	—2.4
12	85.6	36.5	40.4	—3.9
			Mean diff.	—1.8

nic) and of antipyrine was compared following the intravenous administration of the 2 substances to 12 normal humans. The antipyrine space averaged 1.8 liters higher than the NAAP space (Table III).

Use of NAAP for the measurement of body water in the dog. NAAP disappears from the dog at a rate of about 20% per hour. Only about 60% of the NAAP administered to a dog can be accounted for in the urine; the remainder is metabolized. Only the extrapolation procedure, therefore, can be applied to the dog. A dose of about 50 mg per kg will result in plasma levels which can be measured accurately.

Summary. The use of N-acetyl 4-aminoantipyrine (NAAP) in the measurement of body water has been explored. The substance is uniformly distributed in the various tissues in close proportion to the water content, its metabolism is negligible and it is excreted slowly. The volumes of distribution of antipyrine and NAAP were compared in normal human subjects and the values found to agree well. NAAP has certain advantages over antipyrine: it is negligibly bound to plasma proteins; the value for body water may be calculated from a single plasma sample and a urine sample; its colorimetric estimation obviates the need for an ultraviolet spectrophotometer.

Received July 6, 1951. P.S.E.B.M., 1951, v77.

Characteristic Pressure Pulses Recorded with an Esophageal Balloon in Experimental Mitral Insufficiency in Dogs.* (18929)

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This is a study of the pulsation attributable to cardiac activity which can be recorded in the esophagus at the level of the left atrium. It was undertaken to investigate the potentialities of the technic with regard to the diagnosis and possible quantitation of mitral insufficiency. The work of earlier investigators(1-3) along these same lines had not succeeded in conclusively establishing the origin and form of the various pulse waves observed in the esophagus nor was their relationship to events occurring within the left atrium ever clearly demonstrated. Even the work of Taquini(4) and of Puddu and Sibilia(5) was not completely convincing, though it tended to show that a characteristic pattern could be observed in mitral insufficiency in humans. The curves which they obtained, however, showed comparatively minor alterations from those found in normal individuals. Moreover, the patterns did not resemble either the left atrial pressure or volume curves obtained experimentally by Wiggers in dogs with acute mitral insufficiency(6).

All earlier recording had been done with air filled latex balloons attached to elastic rubber tubing. The pressure sensitive element was a rubber diaphragm. Such elastic, air filled systems allow for substantial volume displacement, and therefore record volume changes but do not accurately portray pressure variation. To render the apparatus more sensitive to pressure change the volume displacement permitted in the system was reduced by employing (1) a water filled balloon and completely water filled system; (2) polythene tubing; (3) a relatively rigid pressure

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* Aided by a grant from the Udo M. Reinach Fund.

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sensitive element, *i.e.* Sanborne electromanometer.

It was also observed that as the balloon was distended with successive volumes of fluid, a point was reached beyond which equal increments of volume resulted in decreasing increments of pressure within the system. Therefore, when recordings of the pulse pressure within the esophagus were made, the balloon was distended to that degree at which least increases of volume resulted in greatest increases of pressure. At first latex condoms were used. Later it was found that heavier rubber such as that in rubber gloves gave a more desirable pressure volume relationship.

This is certainly in no sense a rigid, isometric system since both the balloon and the esophagus itself are distensible. However, it is more so than the previously used elastic, air systems and the tracings obtained are surprisingly similar to pressure tracings obtained simultaneously within the left atrium.

Method. The esophageal tube consisted of a length of polythene tubing whose inner diameter was 3.5 mm. One end was sealed and 2 openings into the lumen were cut in the sides of the tube about 3 cm from the sealed end. The rubber balloon was secured to the tube so as to cover both openings. A 3-way stopcock, attached to the open end of the tube, served to connect it with a saline filled syringe and the lead tubing leading to the pressure transducer. All pressures were recorded with Sanborne electromanometers and a 4-channel Sanborne direct writing electrocardiograph. Paper speed was 50 cm per sec. so that each small vertical division represented 0.02 sec.

Pens were aligned so as to write synchronously. Since both the esophageal and atrial pressures were transmitted approximately equal distances through fluid filled rigid tubing, it was felt that no differences in timing existed.

Adult dogs were anesthetized with nembutal. Endotracheal and esophageal tubes were inserted The esophageal balloon was distended with saline as previously described. This resulted in a static pressure within the system of between 9 and 17 mm of Hg. Tracings were then taken at various levels within the esophagus. Intermittent positive pressure respiration was begun, the chest was opened and the balloon was fixed at the level of the left atrium. A left, superior pulmonary vein was ligated distally and a No. 8F cardiac catheter inserted through it into the left atrium. Simultaneous atrial and esophageal pressure curves and lead II of the electrocardiogram were then recorded. Mitral insufficiency was then produced through an incision in the atrial appendage. At the conclusion, the dogs were sacrificed and the hearts examined to verify the degree and location of the lesions.

Results. The curves obtained in 18 normal animals and in 8 following the production of mitral insufficiency are sufficiently similar so that a detailed description of representative samples will illustrate the main features of the entire group.

In Fig. 1 are shown: (A) esophageal pulsation in the normal animal with closed chest, (B) same animal after the pericardium has been opened, (C) same animal following the production of mitral insufficiency. The first pulse wave tracing (A) is typical of that observed in the intact animal. It is characterized by a positive presystolic wave which follows the onset of the P wave of the electrocardiogram by 0.04 to 0.06 sec. It is similar in contour and timing with the wave of atrial systole observed in the atrium itself. This is followed by a series of rapid oscillations, which occur during the first heart sound and probably represents its low frequency components. The exact onset of ventricular systole is difficult to judge exactly. During the period of ventricular systole, the pressure either falls somewhat as in this illustration or remains at the level reached at the end of atrial systole. The curve then rises in a gentle slope and a second set of rapid oscillations is superimposed upon its summit. These are found to occur during the second heart sound and to just precede the fall in pressure which marks the reopening of the mitral valve.

The tracings seen in Fig. 1B show that the opening of the pericardium has not modified the pulse contour appreciably and the same

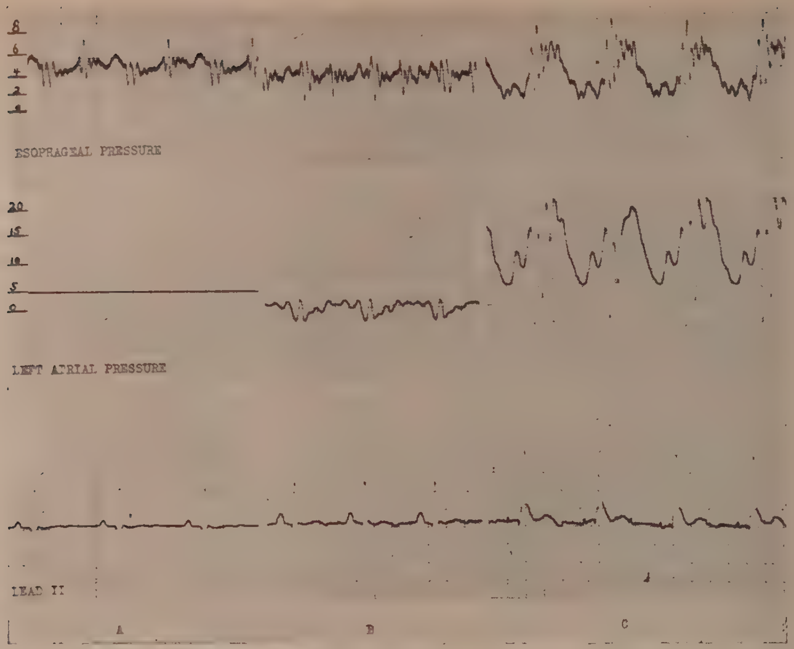

FIG. 1. A. Esophageal pressure tracing and Lead II of electrocardiogram in a dog with a closed chest. B. Esophageal (above) and left atrial (middle) pressure tracings in the same dog after pericardium had been opened. C. Pressure tracings following production of mitral insufficiency, showing characteristic early pressure elevation and sustained systolic plateau.

general features are noticed as in Fig. 1A. The pressure curve obtained directly from the atrium (middle trace) needs no comment. The only noticeable feature is the pronounced wave of mitral closure. This has been observed similarly by Wiggers in dogs(6).

The tracings seen in Fig. 1C, following the cutting of the valve, give unmistakable evidence of mitral insufficiency both in the esophageal (top) and the atrial (middle) pressure tracings. In both, a positive wave of atrial systole is easily identified. The beginning of ventricular contraction is signalled by a sharp rise of pressure beginning 0.04 sec. after the Q wave of the electrocardiogram. The atrial pressure curve displays several marked pressure oscillations during the early phase of ventricular ejection, then a sustained late systolic plateau is seen wherein the atrial pressure maximum is reached. Finally, a rapid fall in pressure marks the opening of the mitral valve. The approximate mean atrial pressure has risen from 0 mm of Hg to about 12 mm of Hg. The pulse tracings obtained synchronously in the esophagus display, with minor differences, the same features as the atrial curve itself. Evidence of the regurgitation jet early in the course of ventricular ejection is shown by the early, sharp elevation of the curve. The late systolic plateau followed by the precipitous descent of the curve also parallel the atrial pressure curve very closely.

There was no attempt made to maintain a constant basal pressure line or zero line in esophageal tracings, and these tracings were arbitrarily centered. The chief interest lay in the determination of the form of the pulse wave and in the calculation of the pulse pressure. This proved to be 13 mm Hg in the atrium and 5.5 mm Hg in the esophagus.

Fig. 2 shows a series of tracings, obtained successively as this animal was bled to death. It is seen that both the mean atrial pressure and the pulse pressure resulting from the regurgitation jet, decline in the successive trac-

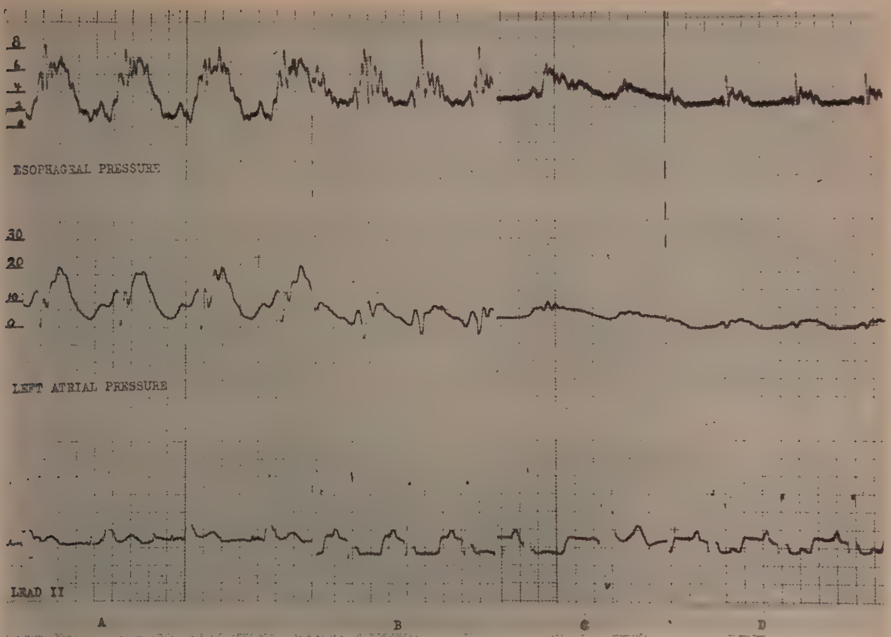

FIG. 2. Tracings A, B, C, and D show successive decline of pulse pressure as the animal was bled to death.

ings. This decline in pulse pressure is almost linearly reflected in the esophageal pulse curve.

In order to demonstrate that the elevated pressure seen during ventricular systole in the esophageal tracings was not somehow due to an arterial impact, the aorta was compressed at its roots. Fig. 3 shows the tracings obtained in another dog in whom mitral insufficiency had been produced, just prior to compression of the aorta (A), during compression (B), and as the aorta was released (C). The compression of the aorta resulted in an increase in the maximum atrial pressure from 16.6 mm of Hg to 110 mm Hg. This is an increase of more than 600%. The pressure in the atrium just prior to ventricular systole increased from —3 mm of Hg to 40 mm Hg. The pulse pressure in the atrium during ventricular systole, therefore, increased from 19.6 mm of Hg to 60.0 mm of Hg. This phenomenon of an increased regurgitation pressure following increase of peripheral resistance was originally shown by Wiggers(6).

The pulse tracings from the esophagus show an elevation of maximum pressure (6 to 15.5 mm of Hg), elevation of the end diastolic filling pressure (0.6 to 6 mm of Hg) and increased pulse pressure (5.4 to 9.5 mm of Hg). There is, unfortunately, no linearity between the atrial and esophageal pressure values at the two levels. The ratio between the atrial and esophageal pulse pressures, for example, is 3.6 to 1 at low pressures and 6.3 to 1 at the high levels. This decreasing responsiveness at high levels of pressure is to be expected, since the balloon becomes progressively more distensable as the pressure within the system rises. However, at low pressure levels there is a rough correlation between the magnitude of the pulse pressure recorded within the atrium and that in the esophagus during ventricular systole. The average pressure ratio observed in the eight animals with mitral insufficiency was 3.3 to 1 with a range of 2.3 to 4.0. The range of pulse pressure variation in the atria was from 7 to 20 mm of Hg and that in the esophagus was 2.5 to 7 mm of Hg.

Summary and conclusions. Pulsations in

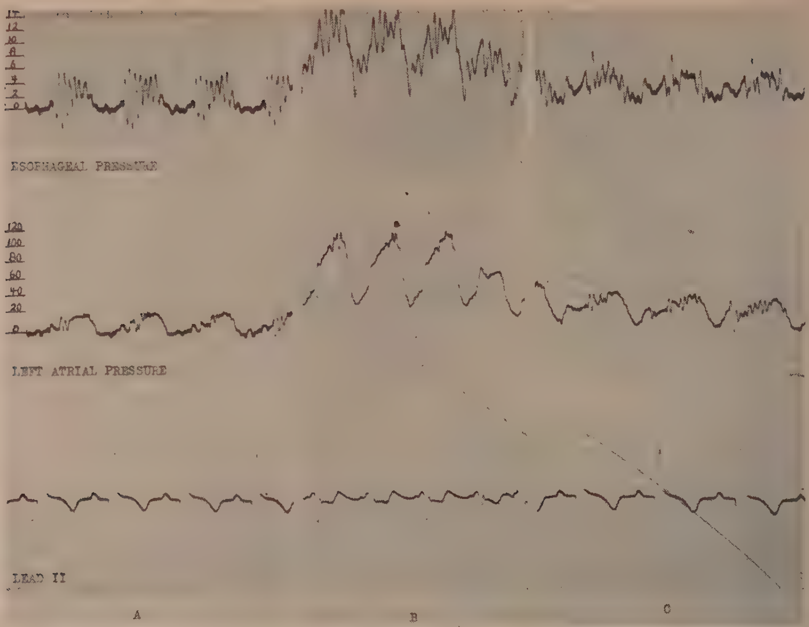

FIG. 3. A. Pressure tracings following production of mitral insufficiency. B. Pressure tracings in the same animal during compression of aorta at its root. C. Tracings after release of aortic compression.

the esophagus at the level of the left atrium and pressure pulses within the left atrium were simultaneously recorded in normal dogs and in the same animals following the production of mitral insufficiency.

An identifiable and consistent pattern in the esophageal tracings was observed in the normal animals. Following the production of mitral insufficiency, characteristic alterations of the esophageal pulse wave contour were observed. These very closely paralleled the pressure tracings obtained simultaneously within the left atrium itself.

It was also possible to detect a fall of the regurgitation pressure which resulted from hemorrhage or a rise which was produced by aortic compression. A ratio of approximately 3 to 1 was observed between simultaneously recorded atrial and esophageal pulse pressures in the dogs with mitral insufficiency.

It is suggested that the contour of the pulse waves of the esophagus at the level of the left atrium, recorded with this technic, closely reflects that of the pressure pulses of the left atrium and that, within limits, a pressure relationship of a quantitative nature exists.

Received July 6, 1951. P.S.E.B.M., 1951, v77.

Mechanism of Acute Hypotension Following Total Body Irradiation.*
(18930)

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Large doses of total body X-ray have been known to result in a shock-like state followed by death. That this true shock-like state is preceded by an acute, moderately severe and rather persistent hypotension is not generally appreciated. The purpose of this paper is to present data on the blood pressures, blood volumes and hematocrits of rats following large doses of total body irradiation; and to determine by the presentation of further data on adrenalectomized and spinal, irradiated and nonirradiated rats the resulting mechanism of acute hypotension.

Methods. All rats used in this study were normal young adult male or female stock white rats. Their weights varied from 339 g to 120 g with the average approximately 200 g. The rats were kept in wire cages and fed the standard diet employed in this institution.

All of the irradiation was given with the same machine at 200 Kv with an inherent filtration of 3 mm Al. In general the rats were irradiated 4 at a time in a small pasteboard box. In all cases following the irradiation, the rats were denied food and water. This was the only time during the course of the experiments that food and water was not readily available to all the animals.

The blood volumes were determined by the method using P^{32}† labeled red cell technic (to be reported). The average adult white rat's blood volume was found to be 6% of body weight.

The hematocrits were determined on whole fresh heparinized blood obtained with the blood volume sample by direct cardiac puncture. Wintrobe hematocrit tubes were used.

The blood pressures were determined in all cases by direct aortic puncture with a 25-gauge needle connected to a saline mercury manometer.

In the bilaterally adrenalectomized animals reported, the adrenalectomies were done singly, and transperitoneally, through lateral abdominal incisions. The first adrenal was removed in each case 5 days before the second in order to insure a nearly normal animal at the time of the second procedure, and to obviate as far as possible the influence of trauma.

The spinal animals were transected at the level of C6 in all cases. The transections were performed with a small pair of dissecting scissors and complete transection was invariably followed by complete paralysis from the low neck down.

Complete autopsies were performed upon all the animals, and H and E sections of the Zenker-fixed material from each organ studied microscopically. The brains and spinal cords were fixed in Zenker's Formol and stained with Phloxine Methylene Blue.

Results. From the data presented in Table II, it can readily be seen that normal rats develop hypotension within 6 hours following 1,500 roentgens of total body irradiation. This hypotension persists for at least 30 hours and is not accompanied by any significant change in either the blood volume or the hematocrit. A total of 23 rats were used in this study.

From Tables IIIa and IIIb it is seen that 2,000 to 2,500 roentgens of total body radiation respectively, result in a hypotension which

TABLE I. Normal Control Rats.

BP mm Hg	Hematocrit	Blood vol (cc)	% Body wt
90	54	12.3	5.6
92	46	15.5	7.4
92	56	13.3	7
95	50	15.5	7
94	52	15.5	7.7
100	50	18.2	7.6
102	54	15.7	6.9

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* This work was done under U. S. Atomic Energy Commission Contract (AT(30-1)-901) with the New England Deaconess Hospital.

† The P^{32} was supplied by the Oak Ridge National Laboratories, Oak Ridge, Tenn.

TABLE II. 1500r Total Body Radiation.

Time interval, hr	BP mm Hg	Hematocrit	Blood vol (cc)	% body wt
6	68	46	14.7	6.95
6	78	47	10.75	4.4
6	72	52	11.85	6
6	77	42	16.65	7.85
6	68	50	16.3	6.95
6	78	48	12.3	6.2
6	78	46	16.3	7.4
6	67	50	17.5	7.45
17	68	38	12	5.85
17	72	44	9.95	4.75
17	64	43	12.2	5.9
17	65	44	9.65	5.35
17	68	41	11.8	5.7
17	68	45	13.25	5.9
17	—	42	14.5	6.15
30	52	45	9.7	4.9
30	58	43	11.15	5.8
30	75	42	12.35	5.8
30	60	43	12.35	5.45
30	48	43	11.35	6.15
30	75	43	11.35	5.7
30	50	40	4.4	2.15
30	58	43	12.25	5.75

TABLE IIIa. 2000r Total Body Radiation—6 Hr Interval.

BP mm Hg	Hematocrit	Blood vol (cc)	% Body wt
—	50	13.5	5.75
73	52	12.75	6
74	49	12.15	5.85
70	50	13.5	6.25
78	50	14.75	6.45
71	49	13.9	6
74	50	12.35	5.85
69	48	13	5.95
70	48	14.35	6.35
71	49	14.65	6.7
70	46	15.35	6.4
70	47	15.2	6.9
68	47	14.65	6.25

TABLE IIIb. 2500r Total Body Radiation—6 Hr Interval.

70	50	10.75	5
75	46	10.9	5.2
68	48	12.35	5.7
84	49	12.3	5.4
72	49	10.9	5.35
73	50	11.3	5.45

is of a similar order of magnitude at the end of 6 hours as that produced by 1,500 roentgens. Nineteen rats were used in this series.

Autopsies upon these rats failed to demonstrate any significant gross changes. The microscopic changes in all rats at the end of 6 hours were similar regardless of the dose group from which they came. These consisted of early destruction, fragmentation and pyknosis of the lymphoid tissue wherever it was found. The mucosal cells in the bases of the crypts of the small intestine showed evidence of early degeneration in the form of nuclear fragmentation, pyknosis and karyorrhexis. The sex cells in the ovaries and testes showed evidence of early disintegration similar to that seen in the lymphoid cells. The H and E bone marrow sections showed evidence of moderate cellular destruction. The lymphocytes show changes similar to those described in the gastrointestinal tract, and the early white cell forms showed some evidence of nuclear pyknosis, the younger cells in general being the most severely damaged. No attempt was made to quantitate this damage. These changes have been described in detail by Warren(2) and more recently by Bloom(3) and the changes mentioned here differ in no way from those described. Other organs and tissues including the brains and spinal cords did not show any abnormalities.

At the end of 17 hours the rats showed a minimal serosal surface hyperemia, but otherwise appeared grossly normal. Microscopically the changes found were identical with those described above, but somewhat more advanced. The germinal centers of the lymphoid tissue were filled with debris and the lymphoid tissue fragments were beginning to disappear. The nervous systems of these animals were not examined.

By the end of 30 hours there was marked destruction and removal of the lymphoid tissue. Some of the mucosal cells of the small bowel showed fairly pronounced fragmentation. There was no sloughing of the mucosa. For the first time, evidence of adrenal cortical change became evident. This consisted of a striking vacuolization of the outer rim of cells of the zona fasciculata. Their cytoplasm was clear and the cells appeared swollen with their nuclei pushed to one side. The H and E bone marrow sections showed moderate evidence of

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1. Montgomery, P. O'B. Data to be published.

2. Warren, S., *Arch. Path.*, 1942, v34, 443; 1943, v35, 121.

3. Bloom, W., *Histopathology of Irradiation*, 1948, v22, I, McGraw-Hill Book Co., Inc., New York.

TABLE IV. Spinal Rats 30 Hr After Cord Transections.

BP mm Hg	Hemato-crit	Blood vol (cc)	% Body wt
85	46	11.6	4.8
80	48	15.9	5
50	50	14.95	5.95
70	51	18.8	6.95
72	48	18.25	6.9
78	52	13.25	5.35
78	47	12.4	4.75
70	39	22.95	7.85
80	56	15.15	5.2
70	37	16.3	6
Spinal Rats 72 Hr After Cord Transections			
40	—	6.4	5.35
68	55	8.95	4.5
70	46	14.7	5.9
80	38	10	6.65
65	41	11.35	5.75
45	51	8.45	4.2
65	51	11.75	5.1
55	52	12.9	6.9

TABLE V. Spinal Rats 1500r Total Body Radiation. BP, BV and hematocrit done 30 hr after cord transections; 6 hr after X-ray.

BP mm Hg	Hemato-crit	Blood vol (cc)	% Body wt
78	57	17.2	5.25
80	52	16.45	7
82	52	18	6.1
70	50	14	5.6
70	51	10.75	6.15
60	45	17.45	5.15
80	53	16.8	5.65
72	47	17.2	6

cell destruction, but no attempt was made to study the cell type or degree of maturation at which the effect was most pronounced. The ovaries showed destruction of ripening eggs and follicular cells, while the testes showed partial and occasionally complete destruction of spermatocytes to the level of the primary spermatocyte. In no case was destruction of ova or spermatocytes complete. Other organs and tissues were normal. The nervous systems of these animals were not examined.

In Table IV are recorded the blood pressures, blood volumes, and hematocrits of the non-irradiated spinal rats. It is at once apparent that at the end of each time interval the results are strikingly similar to those found in the normal total body irradiated rats. Autopsies upon these rats revealed marked distention of the bladder and colon, and the viscera were cool to the touch. No microscopic sections were made.

In Table V are the values for the blood pressures, hematocrits, and blood volumes of the spinal rats subjected to 1,500r total body irradiation. The irradiation was given 30 hours after the transections were performed and the measurements made 6 hours after the irradiation. It is evident that these animals do not differ from their controls in regard to blood pressure, blood volume and hematocrit. Thus, there is no immediate effect upon these values in the irradiated spinal rat, despite the fact that autopsies revealed tissue destruction identical in amount and disposition with that seen in the normal rat given 1,500r total body irradiation.

In Table VI are data showing the effects of bilateral adrenalectomy upon the blood pressure, blood volume and hematocrit. At the end of 6 hours the animals are still hypotensive —a response which the unirradiated rat shows to a similar operative procedure whether or not the adrenals are actually removed—but at the end of 30 hours the blood pressure has returned to relatively normal levels. Autopsies upon these animals revealed no pathological changes.

In Table VII are similar data upon irradiated bilaterally adrenalectomized rats. The 1,500r of total body irradiation was given 30 hours after the bilateral adrenalectomy and the determinations performed 6 hours after the irradiation. The data indicate that these rats behave in a fashion identical to normal

TABLE VI. Bilateral Adrenalectomized Rats. 6 hr interval

BP mm Hg	Hemato-crit	Blood vol (cc)	% Body wt
62	61	17	6.35
74	65	12.9	4.8
85	62	16.45	6
80	56	16.2	5.5
90	56	20	6.5
92	57	23.2	8.3
30 hr interval			
87	51	13.7	6
85	47	14.35	5.85
100	52	13.75	5.7
85	51	14.1	6.2
90	52	18.3	6.85
98	51	16.2	6.1

TABLE VII. Bilateral Adrenalectomized Rats 6 Hr After 1500r TBR and 30 Hr After Adrenalectomy.

BP mm Hg	Hematocrit	Blood vol (cc)	% Body wt
70	54	12.2	4.95
80	50	11.85	4.95
80	51	11.5	4.3
—	54	10.9	3.6
80	52	13.55	5.75
75	53	13.1	4.4
75	56	12.2	4.6
65	52	13.7	4.45
80	56	14.35	4.8
90	51	11.2	4.4
72	51	13.1	4.95

rats given 1,500r total body irradiation. The tissue changes at autopsy of these animals were similar grossly and microscopically to the intact rats 6 hours after 1,500r total body irradiation.

The rats represented by the data in Table I were run as a control group against all the rats receiving irradiation, in order to eliminate any question of blood volume, blood pressure or hematocrit change as a result of depriving the animals of water, and crowding them together in a small box during the time they were irradiated. The animals were put into the radiation box six hours before the determinations were carried out, and left for the same length of time taken by the actual radiation process; they were also deprived of food and water during the six hours preceding the determinations. As shown by the results in Table I no appreciable differences were produced.

Discussion. Normal adult rats develop hypotension within 6 hours following large doses of total body X-ray. This hypotension persists for at least 30 hours. That this hypotension is not a manifestation of clinical shock, as the syndrome is ordinarily defined, is reflected in the failure of either the blood volume or the hematocrit to change during the observed period of hypotension(4).

If this hypotension is not a manifestation of clinical shock, of what then is it a manifestation?

It has been considered by Moon(5) to be due to the toxic effects of the absorption of degenerated intestinal mucous membrane and intestinal contents. That the late true shock picture of irradiation sickness may be due in part to this we do not wish to take exception, but we do not agree that it plays any role in the early acute hypotension following large doses of total body X-ray. Our evidence for this rests on the failure to demonstrate histologically any more than very early evidences of degeneration of the mucosal cells of the gastro-intestinal tract, and the fact that the hypotension continues almost at the same level for the first 30 hours, while histological evidence of cellular destruction both in the gastro-intestinal tract and the lymphoid tissue steadily increases. Furthermore, increasing the dose from 1,500r to 2,500r does not change the response of the blood pressure, and were tissue destruction responsible one would certainly expect more tissue destruction and hence more hypotension with 2,500r than with 1,500r.

The adrenal cortex exerts a profound effect upon the normal blood pressure regulating mechanisms, and removal of the adrenal cortices has long been known to produce severe and constant hypotension. We do not believe, however, that the adrenal cortex plays any significant role in the production of the acute hypotension seen following total body irradiation. The non-irradiated bilaterally adrenalectomized rat goes through a brief period of hypotension due to the operative trauma, which is over within 24 hours. At the end of this time his blood pressure, blood volume, and hematocrit are normal. If he is given a large dose of total body irradiation at this time, he responds as does the non-adrenalectomized irradiated rat. Furthermore, we have failed to demonstrate any histological abnormality in the irradiated animals' adrenals at the end of either 6 or 17 hours following irradiation. The earliest histological change is noticeable only at the end of 30 hours. Thus it is difficult to see how either adrenal failure or abnormal secretion could account for the observed data.

A direct effect of the radiation upon the

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4. Moon, V. H., *Ann. Int. Med.*, 1939, v13, 451.

5. Moon, V. H., Kornblum, K., Morgan, D. R., *J. A. M. A.*, 1941, v116, 489.

cardio-vascular system might account for the hypotension. Indeed this point of view can be defended even in the face of an obvious lack of histological evidence of such damage. An inspection of the data with regard to the spinal rats, however, excludes a possible direct effect upon the cardiovascular system. The 30-hour spinal rat's blood pressure, blood volume and hematocrit are not changed by irradiation. Should there be a direct effect one would expect the irradiated spinal animal to show a more profound hypotension than his non-irradiated control. Thus it is also difficult to conceive of histamine-like substances being the responsible agents, as has been claimed by Painter(6); for again one would expect a further lowering of the blood pressure in the spinal rat following radiation.

It is the remarkable failure of the irradiated spinal rat to differ from his non-irradiated spinal control—in so far as blood pressure, blood volume, and hematocrit are concerned—that lead us to the conclusion that depression of the nervous system is the cause of the immediate hypotension produced by large doses of total body X-ray which are not accompanied by changes in the blood volume or the hematocrit. Additional support for this hypothesis is found in the observations of Prosser *et al.*(7), that the immediate hypotension in rabbits after total body irradiation may be largely guarded against by vagotomy or atropinization; and that it can be antagonized by adrenalin. We have been unable to observe any histological changes in the central nervous system of the irradiated rats by conventional technics. We do not, however, feel that this is any assurance that there has been no change in function. That the autonomic nervous system can be damaged by external radiation has been demonstrated by Suzuki(8). However, as shown by Griffith and Pendergrass(9) the dose must be high, *i.e.* in excess of 800r.

Summary and conclusions. 1. The effects of large doses of total body radiation upon the blood pressure, blood volume, and hematocrit of normal adrenalectomized and spinal adult rats have been studied. 2. From the data presented it appears that the cause of the immediate hypotension following such irradiation is depression of the nervous system.

We wish to acknowledge the technical assistance of Miss Blair Burwell.

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7. Prosser, C. L., Painter, E. E., Lisco, H., Brues, A. M., Jacobson, L. O., Swift, M. N., *Radiology*, 1947, v49, 299.

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Received July 7, 1951. P.S.E.B.M., 1951, v77.

A Simple Assay of Mammalian Pineal Extracts. (18931)

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Amphibian larvae react to feeding with, or injection of, pineal substance with a transient contraction of the skin melanophores. This reaction has been described by McCord and Allan(1) and subsequently by Hogben(2), Engel(3), and others, as occurring in tadpoles only until metamorphosis. At that period the response of the melanophores to pineal extract ceased in the species observed by these authors.

In the following report experiments are described, in which extracts of mammalian pineal bodies were injected into tadpoles and toads (*Xenopus laevis*). The melanophores of the skin of *Xenopus laevis* contract in a specific manner following an injection of pineal extract. This reaction could not be elicited with extracts from any other brain tissue tested. Injection of pituitary extract led to the known effect of expansion of the skin melanophores. The use of Xenopus for a simple assay of mammalian pineal extract is suggested.

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* I wish to thank Dr. E. V. Edwards of the Long Beach Veterans Administration Hospital for the permission to use the laboratory facilities.

1. McCord, C. P., and Allan, F. P., *J. Exp. Zool.*, 1917, v23, 207.

2. Hogben, L. T., Pigmentary Effector System III, *Amphibia*, Oliver & Boyd, Edinburgh, 1924.

3. Engel, P., *Endocrinology*, 1939, v25, 144.

Materials and methods. Seventy toads and 40 tadpoles were kept in glass containers at room temperature (about 21°C). Extracts were prepared from pineal bodies of freshly slaughtered pigs[†] and from one human pineal. The pineals were frozen on dry ice and macerated in a mortar with water, sand, and distilled water or saline. The mixture was then centrifuged and the supernatant fluid used for injection. For most of the work reported here a standard extract (extract A) was prepared by using 500 mg of tissue per cc of distilled water. The supernatant of the centrifuged material (extract A) was injected intra-abdominally, or into the posterior lymph sac of toads. One-tenth cc of the extract was injected into a specimen weighing approximately 50 to 100 g. The amount injected into tadpoles varied with the size of the specimen. Controls were injected with similarly prepared extracts of tissues from various parts of the brain, and with saline and distilled water.

Results. Five to 7 minutes after injection of extract A, the color of the animals started to change from the normal grayish green to a pale pink-white shade. After 30 minutes the height of depigmentation was reached. The toads appeared extremely pale and somewhat translucent. The melanophores remained fully contracted for 30 minutes. After this time, the color of the skin returned gradually to normal. The entire "cycle" (McCord and Allan) from injection to return of normal pigmentation ran a course of approximately one hour in toads and tadpoles alike. The tadpoles appeared colorless and translucent at the height of the reaction, and the change of the stellate melanophores into small spherical bodies could be observed under the dissecting microscope. Animals were used repeatedly for the test, after an interval of several days. The extract from one human pineal body produced an identical reaction. This specimen had been obtained at the post mortem of a white male, 68 years of age, who died of coronary thrombosis.

In higher concentrations than extract A the blanching became more extreme, and the duration of the cycle was prolonged. The highest concentration corresponded to 4,000 mg of pineal tissue per cc of water. Dilution of extract A to 16 mg equivalent of tissue per cc of distilled water led to the characteristic one-hour cycle. With dilution of the extract to a concentration of 8 mg of tissue per cc the bleaching was incomplete and spurious; in further dilutions the bleaching effect could no longer be obtained. The reaction could also be produced by injection of pineal extract into an ablated leg, or by immersion of excised skin into the extract. However, the effect of these procedures was weaker than from injection into the intact animal.

Boiling of the extract did not abolish the activity of pineal extracts upon melanophores. Dialysis for 24 hours against distilled water resulted in loss of activity in this respect.

Summary. (1) The African toad (*Xenopus laevis*) responds to the injection of pineal extract by a contraction of melanophores, in a manner similar to that previously described for amphibian tadpoles, prior to metamorphosis. This response is suggested as the basis for a simple assay of mammalian pineal body. (2) Preliminary chemical studies suggest that the melanophore contracting substance is a relatively stable, low molecular weight compound.[‡]

[†] Courtesy of Mr. M. D. Personnet, Cudahy Packing Co., Los Angeles, Calif.

[‡] Since completion of this report, chemical studies on the melanophore contracting agent have been in progress. Epinephrine has been ruled out. (O.B.)

Received July 9, 1951. P.S.E.B.M., 1951, v77.

Antidiuretic Effect of Non-Renal Venous Congestion on Renal Water Excretion by the Hydrated Rat. (18932)

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An experimental technic for measuring renal excretion after water loading has provided a method of studying the effects of disturbances in normal homeostatic mechanisms in altering renal function. In this study, this technic has been employed to assess the effects of increased venous pressure in altering renal water excretion. This problem has been investigated, using other technics, and has recently been reviewed in detail(1-4). The problem has been a matter of greatest concern in the evaluation of the relative importance of renal and non-renal factors in chronic passive congestion and edema formation.

The present experiments were carried out to evaluate the effects of acute hemorrhage, unilateral nephrectomy, obstruction of lymphatic drainage, and hypophysectomy, in rats in which the venous congestion was produced by one stage complete ligation of the inferior vena cava. A principal finding of note is that the acute passive congestion produced by ligation of the inferior vena cava causes antidiuresis in the presence of a single kidney with unobstructed venous drainage. It is further demonstrated that the hypophysis is not a necessary component in the production of this type of antidiuresis. Finally, the important role of lymphatic drainage in the maintenance of effective circulatory mechanisms in the presence of acute venous obstruction is reemphasized.

Methods and materials. Male rats of the Long-Evans strain, maintained under uniform experimental conditions, fed Diet XIV, were fasted for 18 hours but allowed access to water. The animals were hydrated after various operative interventions by the method described by Birnie *et al.*(5). Each animal received, by gastric intubation, 3 ml tap water/100 sq cm surface area, at hourly intervals for 3 hours. Urine output was collected in metabolism cages and measured at hourly intervals for 3½ hours after the first dose of water. The total urine excretion in this period of time was calculated as % of the water administered. Operative procedures were carried out under ether anesthesia, using clean technics, with the least possible trauma. Inferior vena cava ligations, nephrectomy, and thoracic lymph duct ligations were carried out through ventral abdominal incisions. The anatomical relationships of the renal veins in the rat allowed ligation of the inferior vena cava in different groups of animals at 3 different levels: (1) caval ligation was carried out midway between the right renal vein and the liver (prerenal); (2) caval ligation was carried out midway between the right and left renal veins (interrenal); (3) ligations were made just caudal to the left renal vein (postrenal). Care was taken to include only the vena cava in the ligature, and the animals were examined postmortem to verify that no other vessels or nerve trunks had been included in the ligatures. Left nephrectomy was carried out by ligation of the renal artery and vein distal to the site of entrance into the

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* Assisted by a grant from the National Institutes of Health, United States Public Health Service.

† Acknowledgment for assistance is made to the Institute of Experimental Biology (Dr. H. M. Evans) and to the Department of Biochemistry (Dr. Choh Hao Li). Indebtedness is expressed to Elizabeth Swirnofsky, Roslynn Kenigsberg and to Lorraine Anderson for technical assistance.

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4. Wolf, A. V., *The Urinary Function of the Kidney,* 1950, Grune and Stratton, New York.

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TABLE I. Effect on Water Excretion by the Hydrated Rat of Hemorrhage, Unilateral Nephrectomy, Hypophysectomy, and Thoracic Lymph Duct Ligation, Separately, and Combined with Inferior Vena Cava Ligation.

Procedure	No. of rats	Avg BW, g	Water excreted as % of water administered		Mortality in group (24 hr period)
			Avg % ± SE	(Range)	
Controls					
a. Sham-operated	10	290	72 ± 9	(52–94)	0
b. Acute hemorrhage	4	319	46 ± 4	(34–50)	0
c. External jugular vein ligation	6	296	72 ± 5	(60–83)	0
Inferior vena cava ligation					
a. Prerenal	8	236	.4 ± .4	(0–3)	0
b. Interrenal	8	229	11 ± 3	(1–23)	0
c. Postrenal	4	188	49 ± 4	(37–57)	0
Nephrectomy, unilateral					
a. Left nephrectomy	10	267	70 ± 3	(57–82)	0
b. Left nephrectomy and interrenal caval ligation	9	227	12 ± 5	(1–45)	0
c. Right nephrectomy and prerenal caval ligation	6	264	0	(0)	0
Hypophysectomy					
a. Hypophysectomy	12	132	33 ± 1	(30–35)	0
b. Hypophysectomy and pre- or interrenal caval ligation	9	158	1 ± 1	(0–4)	6 in 24 hr
Thoracic duct ligation					
a. Thoracic duct ligation	6	223	46 ± 5	(34–68)	0
b. Thoracic duct and prerenal caval ligation	6	235	0	(0)	6 in 6 hr

renal vein of the tributary left adrenal vein. The thoracic duct was ligated at the level of the left crus of the diaphragm, at the point of entrance of the duct into the thoracic cavity. Hypophysectomy was carried out in unfasted animals by the parapharyngeal approach. Completeness of operation was verified at autopsy by inspection of the sella under the binocular microscope. Both external jugular veins were ligated at their points of junction with the subclavian veins in an attempt to produce cephalic and cervical congestion. Animals subjected to hemorrhage were bled from the external jugular vein to the extent of 1.5% of the body weight. All animals recovered quickly from the anesthetized state and were subjected to the hydration test within a period of 2-3 hours after operation.

Results. The experimental data are presented in Table I. Normal or sham-operated animals excreted approximately 70% of an administered water load in 3½ hours. The normal rat tolerates ligation of the inferior vena cava at any level caudal to the liver and does not exhibit any obvious signs of shock. Prerenal ligation of the inferior vena cava is a routine first stage procedure in preparation for the operation of evisceration. If, however, such animals (prerenal cava ligated) are subjected to a post-operative hydration test, it is noted that urine formation falls to a negligible quantity in the time interval studied. In such animals, an increased venous pressure exists in the cava, and in the renal, and adrenal veins as manifested by direct inspection and by actual manometric measurement. This increased venous pressure, however, gradually returns to normal as evidenced by the gross appearance of collateral circulatory channels. One also finds a more normal response to the hydration test within a period of 2-3 days postoperatively (unpublished data).

If, on the other hand, cava ligation is carried out midway between the renal veins (interrenal ligation), the increased venous pressure affects only the left kidney and adrenal gland, the right kidney and adrenal gland being situated proximal to the site of ligation. In this preparation, with an unobstructed venous return from the right kidney, 11% of an administered dose of water is excreted. Postrenal ligations of the inferior vena cava result in an excretion of 45% of the administered water in the same time interval.

The above results obtained with different levels of caval ligation suggested that increased renal pressure might be responsible for the failure of diuresis after water loading. To test this hypothesis, similar water loading was carried out in unilaterally nephrectomized rats, with and without caval ligation. It was noted that removal of the left kidney alone did not alter the diuretic response, which was in the control range (70%), whereas if interrenal caval ligation was carried out simultaneously, a procedure which does not obstruct right kidney venous drainage, diuresis fell to a level of the same magnitude (12%) as with interrenal caval ligation alone (11%).

The occurrence of an antidiuresis, in the presence of an unobstructed kidney, suggested that an antidiuretic material (ADH) might be liberated from the pituitary as a resultant of disturbance of the normal homeostatic mechanisms induced by caval ligation. To test this hypothesis, rats were hypophysectomized and subjected to the same hydration test. In the time interval studied, hypophysectomized rats excreted a smaller % of the administered water than did control rats. If, however, hypophysectomy was combined with either prerenal or interrenal caval ligation, excretion of administered water fell to zero. It should be noted, however, that hypophysectomy rendered the rat extremely susceptible to hydration after caval ligation, more than 2/3 of the rats succumbing within 24 hours. Evidently ADH is not an essential element in the production of the marked antidiuresis seen in these animals. It should be noted, however, that these rats were subjected to simple hypophysectomy, *i.e.*, no attempt was made to remove the stalk or related neural tissue known to contain (or secrete) ADH.

In the presence of acute venous obstruction as produced by caval ligation, one of the remaining unobstructed pathways for the return of fluid to the blood is by way of the lymphatic trunks. In order to determine the influence of the pathway, the caval ligation experiments were repeated in animals with ligated thoracic ducts. Depression of water excretion to 46% occurred after hydration of animals with ligated thoracic ducts, but when this procedure was combined with caval ligation, water excretion fell to zero, and strikingly, all animals died within a period of 6 hours. This observation emphasizes the role of the lymphatics in restoring fluid to the effective circulating blood volume in the presence of venous congestion. On the other hand, in control experiments, loss of 30% of the blood volume (arbitrarily estimated at 5% of the body weight) did not produce a remarkable antidiuresis after water loading, the level of excretion being diminished to the level noted after thoracic duct ligation alone. It is noteworthy solely as a control observation that obstruction of the largest venous channels (external jugular veins) draining the head and neck did not produce any degree of antidiuresis.

Discussion. The role of increased venous pressure in relationship to renal excretory activity has been of concern in understanding the mechanisms of retention of sodium and water by the kidney in varied states, as after assumption of the erect posture, exercise, hemorrhage, shock, hypoproteinemia, nephrotic syndrome, and in chronic passive congestion. The present experiments were designed to test the hypothesis that increased caval and renal venous pressure would cause a marked antidiuresis, presumably as the direct result of increase in renal venous pressure, and possibly mediated by increased secretion of ADH by the posterior lobe of the pituitary. Evidence has been presented to show that an antidiuretic material (nature not identified) is present in the body fluids of patients with congestive failure(6). On the other hand, Blake *et al.*(7), have shown that unilateral increase in renal venous pressure in the anesthetized laparotomized dog affected only sodium and water excretion of the affected kidney, thus excluding a humoral

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7. Blake, W. D., Wegria, R., Keating, R. P., and Ward, H. P., *Am. J. Physiol.*, 1949, v157, 1.

or neural pathway for the production of this effect. The mechanism of this sodium and water retention was ascribed to a local action, although no marked changes in glomerular filtration or total renal plasma flow were shown. Contrariwise, Selkurt *et al.*(8), noted, under similar experimental conditions, a progressive reduction in these functions, not sufficient, however, according to these authors, to account for the sodium retention, and, in their opinion, requiring the invokement of mechanisms other than increased renal venous pressure to account for congestive failure. Similar results to those of Selkurt *et al.*, have been obtained by Bradley and Bradley(9), in studying the effects of abdominal compression in man. This subject has recently been reviewed by Smith(1), with the tentative conclusion that the reduction in renal filtration rate cannot be entirely attributed to increase in renal venous pressure, and that other factors, endocrine or hemodynamic, must be invoked to account for the train of events in congestive failure with particular reference to the role of the kidney.

The present experiments provide evidence that the increase in renal venous pressure as produced by ligation of the inferior vena cava(10) is not an obligatory or sole cause of the impairment of diuresis noted, since that antidiuresis occurs to the same degree in animals with a single kidney with unobstructed venous return. Furthermore, in these acute experiments, the antidiuresis is not dependent on the presence of a functioning posterior lobe of the pituitary gland, since it occurs in the hypophysectomized rat with either one or both renal veins obstructed by caval ligation. It is interesting to note that ligation of both external jugular veins in the rat, as might be expected, does not produce sufficient increase in intracranial venous pressure to alter renal function. The present experiments do provide decisive evidence of the necessity for patency of the main lymphatic trunks in the presence of venous obstruction.

The evidence is believed to justify the conclusion that venous obstruction may alter diuretic activity by mechanisms entirely independent of (and additional to) local effects produced by increased renal venous pressure. Hemodynamic effects are more likely important in terms of decrease in effective circulating blood volume, although neurogenic and humoral effects (other than from the posterior lobe of the pituitary) have not been negated by the experimental data presented.

Summary. (1) Water excretion by hydrated rats has been studied to ascertain the alterations after ligation of the inferior vena cava at 3 levels in relationship to the renal veins. In addition, the effects of unilateral nephrectomy, hypophysectomy and thoracic lymph duct ligation, and various other control procedures have been observed. (2) The antidiuretic effect of inferior vena cava ligation in the hydrated rat is shown to be independent of increased renal venous pressure, and is also shown to occur in the absence of the pituitary gland. (3) The critical importance of the lymphatic pathway in maintaining an effective circulating blood volume in the presence of acute venous obstruction is demonstrated by showing the lethal effects of hydration in animals with combined caval and thoracic duct ligation, procedures which are separately tolerated by the rat.

Received July 10, 1951. P.S.E.B.M., 1951, v77.

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The Lability of Thiamine in Certain Purified Rations.* (18933)

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Hankes and Elvehjem(1) described the development of a nervous condition in rats upon feeding a low protein, niacin-free, ration supplemented with DL-phenylalanine and DL-methionine for a period of 10-12 weeks. The symptoms included an arched back, oscillation of the head, loss of equilibrium, and convulsions which were followed by death in 36 to 50 hours. It was recognized that the symptoms resembled a thiamine deficiency but the following conditions, adequate supply of thiamine in the ration (.2 mg %), delayed development of the symptoms, and complete prevention of symptoms when 18% of casein was used or when niacin or tryptophan were supplied from the beginning of the experiment, suggested a separate and distinct syndrome. When these studies were continued, considerable variation was encountered in the production of the symptoms and finally injections of thiamine were tried. The injection of a rather large single dose (80 μg) alleviated the convulsions and the continued administration of the vitamin restored normal growth in the animals.

We wish to report in this paper the rapid destruction of thiamine in some of the rations used and the remarkable stabilizing effect of succinylsulfathiazole. Any specific effect of phenylalanine and methionine in such rations cannot be determined until the level of thiamine is more accurately controlled.

Experimental. Forty-five male weanling rats of the Sprague-Dawley strain were placed in separate screen-bottomed cages and divided into 9 groups of 5 animals each. All were placed on a thiamine-free basal ration for 1 week. The different groups were then fed the rations listed in Fig. 1. The basal ration consisted of sucrose 79.8%, corn oil 5%, casein† 9%, salts IV(2) 4% and DL-methionine 0.2%. A niacin-free vitamin mix‡ diluted with sucrose was added at a 2% level. In series 2 the thiamine was omitted from the vitamin mix. Thus the animals in series 1 received the same level of thiamine (0.2 mg/100 g ration) and in the same manner as those in the previous studies. The rations for groups 2, 4, 6 and 8 were also supplemented with 1.5% succinylsulfathiazole in order to minimize any synthesis of thiamine in the tract. Kratzing and Slater(3) have shown that this drug does not contribute to the thiamine economy of the rat.

All the animals in series 2 received the thiamine by injection. The thiamine solution was prepared weekly and contained 10 μg per ml. Initially each animal received 10 μg daily but this was reduced to 8 μg at the end of the second week and to 5 μg at the end of the fifth week. This level was then maintained until the end of the experiment.

The thiamine content of the rations and the livers from the rats in series 2 was determined by the thiochrome method described by John-

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the Nutrition Foundation, Inc., New York, and the Robert Gould Research Foundation, Inc., Cincinnati, Ohio. We are indebted to Merck and Co., Inc., Rahway, N. J., for the crystalline vitamins; to the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for synthetic folic acid; and to Abbott Laboratories, North Chicago, Ill., for Haliver oil.

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‡ Vitamins were added as a dry mix and provided per 100 g of ration; thiamine-HCl 0.2 mg, riboflavin 0.3 mg, pyridoxine 0.25 g, calcium DL-pantothenate 2 mg, choline chloride 100 mg, inositol 10 mg, biotin 0.01 mg, and folic acid 0.02 mg. In series 2 the thiamine-HCl was omitted. Periodic analysis of the mix showed that the thiamine destruction was negligible over a 10-12 week period. Halibut liver oil fortified with vitamin K and vitamin E was administered by dropper once weekly.

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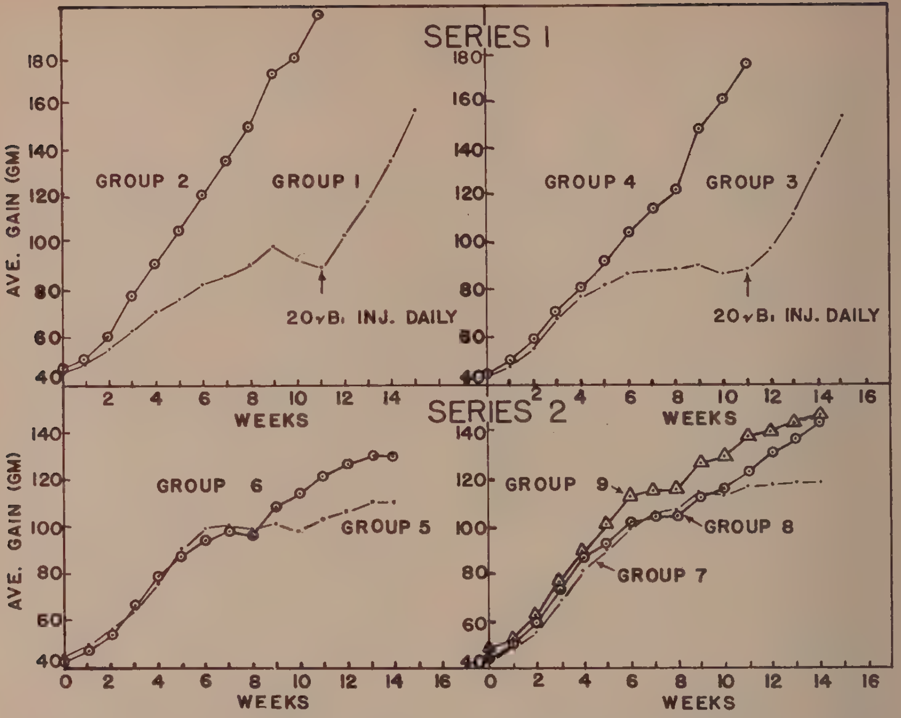

FIG. 1. Growth response of rats fed a niacin-free 9% casein basal ration under the following conditions: Series 1—thiamine incorporated directly into the ration. Group 1, basal ration; group 2, basal ration + 1.5% sulfasuxidine; group 3, basal ration + 0.2% DL-phenylalanine; group 4, basal ration + 0.2% DL-phenylalanine + 1.5% sulfasuxidine. Series 2—5 μg thiamine injected intraperitoneally daily. Group 5, basal ration; group 6, basal ration + 1.5% sulfasuxidine; group 7, basal ration + 0.2% DL-phenylalanine; group 8, basal ration + 0.2% DL-phenylalanine + 1.5% sulfasuxidine; group 9, basal ration + 0.2% DL-phenylalanine + 1.5 mg % niacin.

son(4) and by Gyorgy(5). Extraction of the samples was carried out in most cases in 0.1 N H_2SO_4, with steaming for 30 minutes. In addition to the steaming, the livers were incubated with pepsin and takadiastase for 18 hours after adjusting the pH to 4.5 with sodium acetate.

Results and discussion. The growth data are shown in Fig. 1. The rats in group 1, control, and group 3, with added DL-phenylalanine, grew slowly until the eighth or ninth week, at which time the gains in both groups ceased. By the eleventh week all the animals in both groups had developed convulsions. When these animals were given a single injection of 80 μg of thiamine, all were cured of the symptoms except one animal in group 3 which died upon injection. Continued intraperitoneal injections of 20 μg of thiamine daily restored normal growth and appearance of the animals in both groups. In contrast to the poor growth exhibited by the animals in groups 1 and 3, those in groups 2 and 4 grew remarkably well. Food consumption records showed no significant differences between the groups during the first 3 weeks with an average consumption of 5-6 g of ration per rat per day. By the eighth week the animals in groups 1 and 3 were eating about 4.5 g and those in groups 3 and 4, almost 10 g per day. The only difference in the rations was the presence of sulfa drug. The effect could not

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TABLE I. Results of Thiamine Analysis of Rations Used in Series 1* and Livers from the Rats in Series 2.

	Series 1		Series 2	
Supplement	Group No.	B_1 content of ration after 6 days, $\mu g/g$	Group No.	B_1 content of liver. Avg of 3 animals, $\mu g/g$ liver
None	1	.52	5	1.32 ± .03†
1.5% sulfasuxidine	2	1.9	6	1.20 ± .09
0.2% DL-phenylalanine	3	.56	7	1.35 ± .19
” ” + 1.5% sulfasuxidine	4	1.7	8	1.30 ± .13
0.2% DL-phenylalanine + 1.5 mg % niacin			9	1.21 ± .14

* Determinations made on rations 6 and 8 showed no fluorescence, so the sulfasuxidine was not contributing to the values of rations 2 and 4.

† Standard error $\sqrt{\dfrac{\Sigma d^2}{n(n-1)}}$.

be due solely to alterations in the intestinal flora which might allow more thiamine to be available to the host. If this had been true, groups 6 and 8 of series 2, receiving identical rations except for a daily injection of a limited amount of thiamine, should have shown a similar growth response. The increased growth observed in groups 6 and 8 over that observed in groups 5 and 6 may have been due to an increased availability of tryptophan resulting from the effect of the sulfa drug since the growth response was quite similar to that of group 9 which received 1.5% of niacin in the ration. The rate of growth of all the rats in series 2 was undoubtedly limited by the lower supply of thiamine. These animals consumed only 5-6 g of ration per day. Further evidence that the sulfa drugs did not alter the availability of thiamine is shown in Table I. The thiamine levels in the livers of the rats from the different groups are very similar.

In series 1 the rats on the niacin-free basal control ration developed convulsive symptoms as frequently as those receiving added DL-phenylalanine. In series 2 no increased tendency toward the convulsions was exhibited by the rats in group 7 which received the phenylalanine. Thus, it appears that phenylalanine does not directly increase the demand for thiamine.

The reason for the superior growth response of the animals receiving sulfasuxidine became evident when the ration was analyzed for thiamine. Examples of typical analyses are shown in Table I. The original rations contained 2 μg of thiamine per g ration. While 70-75% was destroyed in the non-sulfasuxidine mixture, nearly all of the vitamin was recovered from the rations containing the drug. Thus the animals of groups 2 and 4 received from 15-20 μg of thiamine per day, while animals of groups 1 and 3 received less than 5 μg per day. Actually the amount must have been considerably less than 5 μg since those in series 2 received 5 μg and showed no signs of convulsions. The protection afforded by the sulfa drug does not seem to be one of bacterial inhibition in the mixed ration for as much as 50% destruction can be shown in a matter of hours after preparing the ration. This rapid destruction is also shown by a lack of growth response when a freshly mixed ration is fed. Very little depletion of the vitamin occurs during storage of the vitamin mix over long periods. In an effort to determine whether the method of extraction was exaggerating the destructive effect, samples were extracted with cold water or else sulfasuxidine was added just before the acid extraction. However, analyses made in this way within a short time after preparing a fresh mix indicated up to 50% destruction which increased to as much as 80% within 2 weeks.

The exact cause of this vitamin destruction is not known, but present information indicates that the salts IV used in the ration provides the destructive agent or agents. Preliminary work has shown that potassium iodide in relation to the copper sulfate and ferric citrate may provide the destructive agent. De Ritter and Rubin(6) showed that ferrous iron would destroy thiamine under conditions of acid extraction, but that this destruction could be avoided by the addition of cystine to the extraction solution. It is of some interest to note that when L-cystine replaced the DL-methionine as the sulfur amino acid supplement, no convulsions were observed and growth was considerably improved. However, cystine added to the extraction solution or to the ration does not improve the recovery of the vitamin.

The data presented in this paper are based on a limited number of animals but similar results have been obtained with many other groups of animals. In all cases when a reduced growth response has been obtained a definite loss of thiamine in the ration as revealed by chemical analyses has been demonstrated. While more work is needed to establish the exact mechanism of the destruction and the protective action of the sulfa drug, it seemed advisable to bring these effects to the attention of other workers. Since the lability of thiamine has been encountered in rations containing only 9% casein and no added niacin or tryptophan, one might conclude that protein and tryptophan or niacin may have some protective effect. However, it is more likely that a deficiency of tryptophan or niacin may reduce the food intake sufficiently to precipitate a thiamine deficiency when a significant portion of that added to the ration is destroyed. These relationships may be studied further when the level of the thiamine in the ration can be accurately controlled.

Summary. The "nervous syndrome" condition produced in rats after 10 weeks or longer on a low protein, niacin-free regimen has been shown to respond to thiamine supplementation. Complete protection from the convulsions was affected when sulfasuxidine was added to the ration. Analyses of the rations for thiamine showed that as much as 70% of the vitamin was destroyed in the non-sulfasuxidine ration, while very little destruction occurred in the ration containing the drug. The addition of 0.2% DL-phenylalanine did not increase the thiamine requirement.

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Received July 10, 1951. P.S.E.B.M., 1951, v77.

Use of Cortisone in Treatment of Experimental Frostbite. (18934)

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It has been reported that cortisone and ACTH therapy has been beneficial in the treatment of severe burns(1,2). Since frostbite resembles burn injuries in some respects, cortisone has been used in the treatment of frostbite casualties in the Korean conflict. It has been demonstrated that the adrenal glands become enlarged in rabbits following local cold injury, and in severe frostbite pathologic changes have been observed in the cortices(3). However, as far as we know, no clinical or experimental evidence has been presented to support the use of cortisone in local cold injuries. These experiments were performed to determine the effect of this drug on the extent of frostbite necrosis.

Materials and methods. The experiments were performed on 65 male albino rabbits of more than 2,500 g body weight. Two degrees of local cold injury were induced, one by exposure of a hind leg to an alcohol bath at –12°C for 30 minutes for the determination of muscle necrosis, and the other at –15°C for 30 minutes for the determination of skin as well as muscle necrosis. We have shown in

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TABLE I. Muscle and Skin Necrosis After Local Cold Injury.

Exposure (°C)	Test animals	Results Muscle necrosis, %	 Skin necrosis, cm^2
–12° for 30 min	18 treated with cortisone	24.5 ± 4.6	
	17 controls	26.7 ± 4.1	
–15° for 30 min	15 treated with cortisone	61.4 ± 7.2	11 ± 2.5
	15 controls	57.1 ± 7.1	11 ± 3.7

previous experiments that these exposures result in injuries which are critical for muscle and skin, respectively(4).

Thirty-five animals had one hind leg exposed to –12°C for 30 minutes, of which 18 were treated with cortisone* intramuscularly. Two and one-half mg were given intramuscularly in the thigh of the unexposed leg twice daily for 2 days beginning with the day of exposure followed by 1.5 mg twice daily for 5 days. Seventeen rabbits did not receive cortisone and served as controls. All animals were sacrificed 7 days after exposure, and the extent of muscle necrosis was determined in percent of the total weight of the leg muscles as described previously(5).

A second group of 30 animals was exposed to –15°C for 30 minutes to determine the extent of skin necrosis and also the extent of muscle necrosis. Fifteen of these received 2.5 mg cortisone intramuscularly twice daily for 2 days beginning with the day of exposure followed by 1.5 mg twice daily for 4 days. Fifteen did not receive cortisone and served as controls. All animals were sacrificed after 6 days. The area of necrotic skin was measured in square centimeters.

The exact procedures of freezing and measuring skin and muscle necrosis have been described in other papers(4-6). Except for cortisone administration, all animals received the same care after exposure. The frozen legs were coated with 3% sulfamylon ointment, dressed, and left to spontaneous rewarming in air at room temperature. The animals were placed in individual cages in the air-conditioned animal room and dressed daily. The average results are given with the standard deviation of the mean.

Results. None of the 35 rabbits exposed to –12°C for 30 minutes for the determination of muscle necrosis died before the end of the experiment, and all developed muscle necrosis. Three cortisone-treated and 5 control animals developed small areas of skin necrosis. The percentage of muscle necrosis in the two groups is given in the table. The results in the cortisone-treated and control animals are not significantly different.

Of the 30 animals exposed to –15°C for 30 minutes for the determination of skin and muscle necrosis none died before the end of the experiment. Four cortisone-treated rabbits and 5 control animals did not develop skin necrosis but were included in the calculations. All developed extensive necrosis of muscle.

As shown in Table I, neither the extent of skin necrosis nor the percentage of muscle necrosis differs in the control and cortisone-treated groups.

Conclusions. Cortisone in the doses used in these experiments was not effective in reducing the extent of muscle or skin necrosis in rabbits after 2 standard local cold injuries.

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* Cortone Acetate, Saline Suspension. Merck and Co., Inc.

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Received July 10, 1951. P.S.E.B.M., 1951, v77.

Production of Dietary Necrotic Liver Degeneration Using American Torula Yeast. (18935)

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Dietary necrotic liver degeneration† was first recognized as a deficiency disease when Weichselbaum maintained rats on a cystine free diet(1). It is entirely different from fatty liver and cirrhosis, as first pointed out by Daft, Sebrell, and Lillie(2).

Necrotic degeneration can be produced by a wide variety of rations (Table I). All of these diets seem to be deficient in vitamin E and low in sulfur containing amino acids, especially cystine. The deficiency can be induced by diets which contain pure amino acids in place of protein, as reported incidentally by DuVigneaud, Dyer and Kies in a paper dealing with transmethylation(3). The protective action of cystine, discovered by Weichselbaum, was independently confirmed by Daft, Sebrell, and Lillie(2), Hock and Fink(4,5), Glynn, Himsworth and Neuberger (6), and others.

It has been shown since that dietary necrotic liver degeneration in most instances develops only when cystine and vit. E are insufficiently supplied at the same time and that each of these substances alone is able to protect(7). The significance of vit. E as a protective agent against necrotic liver degeneration was discovered by Schwarz(8).‡ The finding was confirmed by Gyorgy(9) and by Himsworth and Lindan(10) and Goettsch(11). It is possible that at least one other not yet identified factor may be protective against the disease(12). The question of whether necrotic liver degeneration, induced by different diets, constitutes an indivisible entity or whether several kinds or types of necrotic degeneration have to be distinguished deserves further investigation.

The deficiency can be induced in growing rats when the protein requirement is met by large amounts of yeast in purified diets(4,5). However, only certain yeasts have this effect, whereas others do not. Most European yeasts examined produced the deficiency in almost 100% incidence(7) whereas most American yeasts did not induce any degeneration or produced the deficiency only in a small percentage of animals after extended experimental periods. The lesions have been found using

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* Research done under a Special Research Fellowship, U. S. Public Health Service.

† "Degeneration" is defined as progressive deterioration of tissue in which its vitality is diminished, whereas "necrosis" means death of a circumscribed portion of tissue. "Dietary necrotic liver degeneration" is applied in this paper to define a deficiency disease which in previous publications has been called "hemorrhage"(1), "hemorrhage and necrosis" (2,6, etc.) or "liver degeneration"(7,8). The defect is characterized by a degenerative change which eventually leads to acute extensive liver necrosis, hemorrhages and death.

‡ The journal was republished in 1945 in the United States under the auspices of U.S.O.A.P.C., by J. W. Edwards, Ann Arbor, Mich.

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TABLE I. Diets Producing Necrotic Liver Degeneration in Rats.

Cystine-free (corn starch 80%, dry milk powder 16%)	Weichselbaum(1)
Cystine-free (amino acids in crystalline form)	DuVigneaud, Dyer and Kies(3); Glynn, Himsworth and Neuberger(6)
Low casein (10% or less)	Daft, Sebrell and Lillie(2); Gyorgy and Goldblatt(19); Hove and Harris (16)
Casein VI (alkali-treated, 15%)	Schwarz(17)
Yeast	Hock and Fink(4,5); Glynn and Himsworth(18); Schwarz(7); Gyorgy and Goldblatt(20)
High cod liver oil	Schwarz(21)
Potato protein, pea protein, cereal protein, Gelatin (in various combinations)	Hock and Fink(22)
Soy bean meal (raw, fat extracted)	Matet, Matet and Fridenson(23)

18% or less of American bakers yeast(13,14). This amounts to about 7% of protein in the diet which is inadequate in various amino acids. The resulting damage is complicated by these defects(15).

The peculiar discrepancy between American and European yeasts in regard to necrotic liver degeneration is not due to differences in variety, since very different strains of yeast have the same property and apparently not due to technical differences during production. It appeared to us, however, that the reason for the dissimilarity might be found in differences of media applied for the cultivation of the yeasts, grain products, *i.e.* corn steep liquor being more important ingredients in the U.S.A., whereas beet molasses, sulphite liquor, and wood hydrolysate are more widely used in Europe. Following this line of thought we found that torula yeast, grown in the United States in a simple medium (sulphite liquor) can be used for the induction of liver degeneration as well as European samples.

Experimental. Weanling rats of the Sprague-Dawley strain (National Institutes of Health Colony), weighing usually 35 to 40 g were divided into groups of 10 animals of equal weight, sex, and litter distribution. The animals were housed in individual elevated wire mesh cages. Weights were recorded once or twice weekly. Diets and tap water

TABLE II. Composition of Diets.

	Control diet	Torula diet	Yeast K diet
Casein*	15	—	—
Yeast	—	30	30
Sucrose†	74	59	59
Salt‡	5	5	5
Lard, Vit E-free§	5	5	5
Vitamin powder‖	1	1	1

* "Vitamin free," alcohol extracted.

† Commercial, regular grade.

‡ NaCl 54 g, $Na_2HPO_4 \cdot 7H_2O$ 91.5 g; $CaHPO_4$ 145.0 g; KH_2PO_4 258 g; Ferric citrate ($FeC_6H_5O_7 \cdot 3H_2O$) 32.5 g. Ca lactate ($(CaCO_3H_5O_3)_2 \cdot 5H_2O$) 349 g; $MgSO_4$ 70 g; KJ·715 g. After mixing, the following are added: $MnSO_4 \cdot 7H_2O$ 325 g; $ZnSO_4 \cdot 7H_2O$ .4959 g; $CuSO_4 \cdot 5H_2O$ .285 g.

§ Vitamin E-free animal fat, stripped by molecular distillation (Distillation Products Inc., Rochester, N. Y.).

‖ Lactose 97.861 g, thiamine HCl 40 mg, riboflavin 25 mg; pyridoxine HCl 20 mg; D-calcium pantothenate 44 mg; choline chloride 1 g; niacin 1 g; menadione (2-methyl-1, 4-naphthoquinone) 10 mg.

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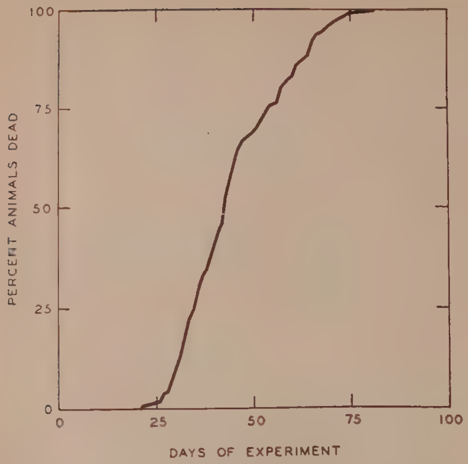

FIG. 1. Death from dietary liver degeneration. (Average of 15 experiments, Aug. 17, 1949-May 17, 1950.)

were given *ad libitum*. The diets (Table II) were prepared in dry form and fed in porcelain cups. A standard diet containing 15% purified casein was used for comparison. In yeast-diets, casein was replaced by 30% yeast, the difference of 15% was compensated for by a corresponding reduction in sucrose. The torula yeast was obtained from the Lake State Yeast Corp., Appleton, Wis.; Yeast K (dried, debittered brewer's yeast) was purchased from Anheuser Busch, Inc., St. Louis, Mo.[§] The common water soluble vitamins and vit. K were incorporated routinely irrespective of the vitamin content of the yeast (Table II). Fifty γ of crystalline vit. A alcohol and 1 γ of crystalline vit. D_2 dissolved in 0.025 cc of ethyl laurate were given orally once weekly.

A thorough autopsy was performed on each animal. The effect of protective supplements was evaluated by comparing the average reciprocal survival time (multiplied by 100 in order to obtain suitable figures). This presents a mathematical expression for the velocity of the development of death. For example, for an animal surviving 25 days, the reciprocal is $\frac{1 \times 100}{25} = 4.0$. For animals surviving indefinitely the figure O is assigned. Average values for groups were calculated and compared.

Results. In animals on 15% alcohol extracted "vitamin free" casein necrotic liver degeneration has never been found either in these or in other extensive tests. When the torula diet was fed, the rats succumbed to necrotic liver degeneration in 100% incidence. Two groups of 10 animals on the yeast K diet grew well, and were sacrificed after surviving 70 and 145 days respectively. Their livers were normal.[¶]

Average results on torula diet. In 15 consecutive experiments with 14 groups of 10 and 1 of 12 animals on the torula ration, 145 out of 152 animals died with the necrotic liver disease; the other 7 rats died with complications (6 pneumonias, 1 kidney degeneration) during the test. The average survival time on the torula diet was 45.5 (22-83) days. The time mortality curve (Fig. 1) shows that death occurred, starting at the 22nd day, at a rather constant rate until about the 48th day. From there on the mortality rate declined.

Effect of L-Cystine. When L-cystine was added to the torula ration, increasing protection was afforded by increasing amounts (Table III). On a .1% L-cystine supplement all animals died, survival being slightly prolonged, as indicated by a 17.5% reduction of the average reciprocal survival time. Almost complete protection was provided when the ration was supplemented by .5% L-cystine from the start, or when .5 or 1.0% were added at the 26th day of the experiment.

Protective effect of vitamin E. The strong protective action of vit. E, formerly demonstrated in experiments with European yeast diets(7), is shown in Fig. 2. A supplement of 5 mg % alpha tocopherol acetate in

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[§] It is emphasized that the results reported in this publication do not allow any interpretation regarding the usefulness of various yeasts in animal nutrition since these materials were fed as the sole source of protein in highly purified, vit. E deficient rations.

[¶] Two other American yeasts (yeast G, cultured, primary dried *Saccharomyces cerevisiae*, Anheuser-Busch, and an USP dried brewers yeast of unidentified origin) have been tested for their ability to produce necrotic liver degeneration using a system somewhat different to that described herein. They, like yeast K, failed to produce the defect.

TABLE III. Effect of L-Cystine.

% L-cystine supplement in diet	No. of animals	Dead due to liver degen.	Avg reciprocal survival time (×100)		% reduction of reciprocal survival time§
			a. Controls‡	b. Experimental group	
.1	10	10	2.3	1.9	17.5
.2	9	6‖	2.3	.74	67.8
.5	9	3¶	2.08	.36	82.7
.5*	10	1¶	1.94	.25	87.1
.5†	7	1**	2.49	.26	89.4
1 †	9	1**	2.49	.19	92.5

* Pairfed to controls. † Supplementation started at 26.day. ‡ Littermates on torula diet with no cystine supplement. § (a-b) in % of a. ‖ Terminated 100th day. ¶ Terminated 112th day. ** Terminated 177th day.

the torula diet prevented the disease in all instances. With 1.25 or 2.5 mg % degeneration occurred only after very prolonged experimental periods. Surviving animals killed at the end of these tests had macroscopically normal livers.

Description of the disease. Rats on the torula diet stopped growing after 3-4 weeks, without displaying any other symptoms. After 22 to 83 days they suddenly lapsed into a fatal period which took but a few days or hours. At the beginning of this striking phase the animal remains quiet though trembling, and the body feels cool. Sometimes short attacks of hyperactivity can be seen. There is a slowing of reflexes and gradual loss of consciousness until a deep coma is reached which is accompanied by occasional convulsions. This stage persists for one to several hours, during which time the heartbeat and breathing fade; the transition from life to death occurs slowly and quietly.

Upon dissection, the most outstanding changes are seen in the liver (Fig. 3). A

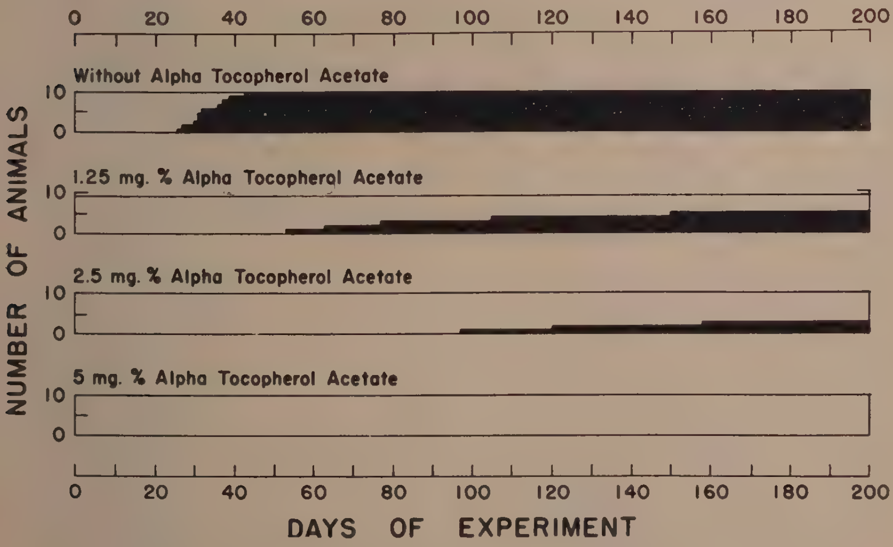

FIG. 2. Inhibition of liver degeneration by alpha-tocopherol acetate.

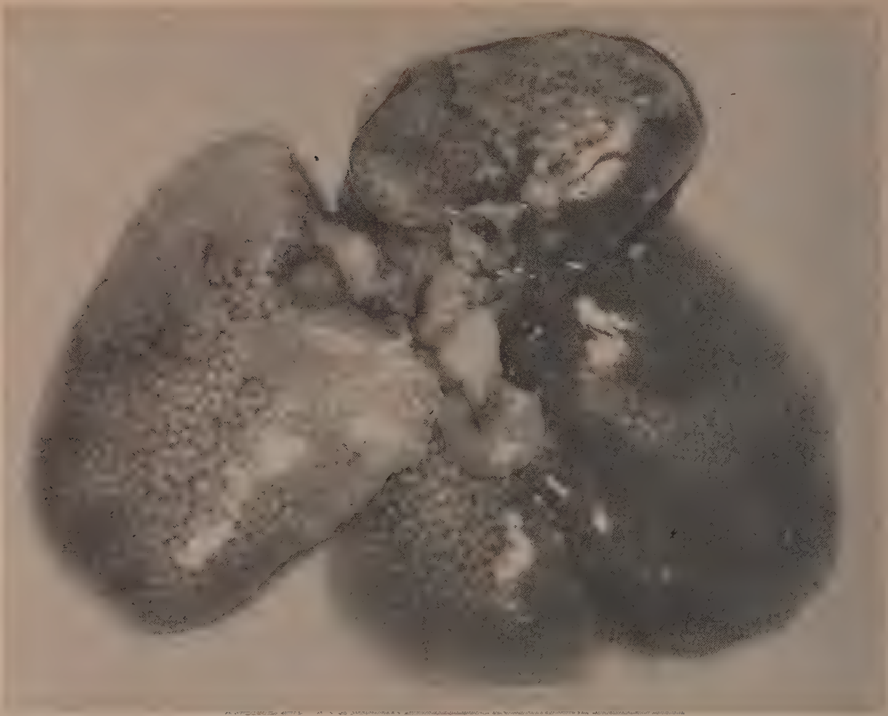

FIG. 3. Dietary necrotic degeneration of rat liver.

macroscopic description and histological analysis will be published separately.

The severe damages of the liver, which are precipitated in the short terminal period, are apparently the reason for the sudden death. In many of the animals degenerative changes can also be seen in the forestomach and the kidneys, and acute lesions are frequently found in the lung and the lymphatic system.

Ulcers of the forestomach of pinpoint to millimeter size (Fig. 4) occurred in about 51% of the rats. The lesion has been described previously(16), it is prevented by vit. E and seems to belong to the cycle of changes pertinent to dietary necrotic liver degeneration.

The kidneys of the rats which died displayed a whitish intercorticomedullary zone in about 87%. This white, often incrusted, area resembles that seen after the feeding of adenine, and might be caused by accumulation of dioxyadenine or similar products, due to the large amount of nucleic acids present in the yeast. Macroscopic defects of the cortex were rare.

Changes which are directly connected with the terminal phase of life occur in the lung. Acute lung edema was present in about 40% of animals, congestion and hemorrhages were seen in most of them. Hemorrhage also appeared in the gastrointestinal tract. The abdominal and mediastinal lymph nodes were hemorrhagic in many cases.

Summary. American torula yeast, when used as the sole protein source in Vit. E free diets, induced dietary necrotic liver degeneration in rats in the same manner as reported for European yeasts. Other American yeasts did not produce the defect. With 30% torula yeast in the diet the incidence of liver degeneration in 15 consecutive experiments was 100%, the average survival time was 45.5 days.

L-Cystine reduced the incidence of the defect considerably; however, even 1.0% did not give full protection when supplementation

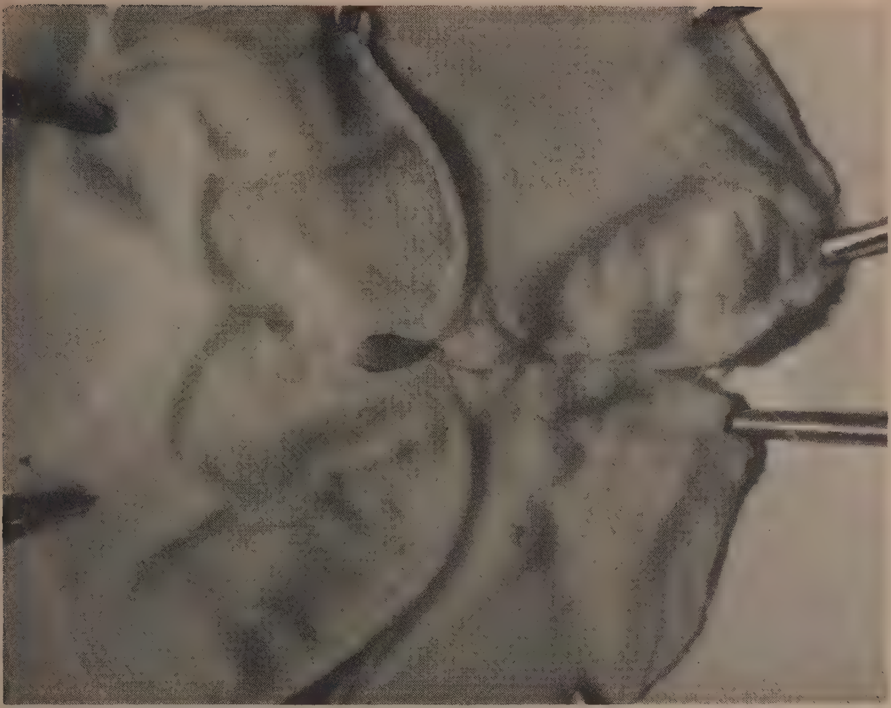

FIG. 4. Ulcers in forestomach of rat on torula diet.

started at the 25th day. Five mg % vit. E prevented the defect completely.

Necrosis of the liver developed very suddenly after an experimental period of 22 to 83 days and was accompanied by kidney lesions, forestomach ulcers, acute lung edema and hemorrhages in the lungs, in the lymphatic tissues and occasionally in the intestine.

Acknowledgement is made to Mr. Louis E. Lowman and Mr. William Beane for technical assistance.

Received July 16, 1951. P.S.E.B.M., 1951, v77.

In Vitro Effect of Ethyl Alcohol on Respiration of Rat Liver and Kidney Slices.* (18936)

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In studies (unpublished) on the *in vitro* effect of testosterone on tissue respiration, ethyl alcohol was used as the solvent for the androgen. It seemed important, therefore, to determine the influence of various concentrations of alcohol on the *in vitro* respiration of liver and kidney slices.

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* This investigation was supported by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

The data in this report have been mentioned in a Symposium on Influence of Hormones on Enzymes June 5 and 6, 1951 sponsored by the New York Academy of Sciences.

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Procedure. The oxygen consumption of liver and kidney slices from adult male rats from our colony was measured at 37.5° by the Warburg technic with Summersen flasks and manometers. The tissues were sliced with a Stadie-Riggs microtome(1), weighed on a Roller-Smith Torsion balance and transferred to the respirometer flasks containing Ringer's solution buffered to pH 7.4 with N/150 phosphate(2). The center well contained 0.2 ml of 5% potassium hydroxide and a cylinder of filter paper. The alcohol was added just prior to the attachment of the flasks to the manometers. The system was oxygenated for 15 minutes, equilibrated for another 15 minutes and then readings made at 20-minute intervals for the first hour and at 30 minutes for the next hour. The tissues were removed at the end of the experiment by filtration on glass cloth rinsed with distilled water, dried at 95 to 100°C on tared discs of aluminum foil, weighed and analysed for nitrogen by the micro-Kjeldahl procedure. Calculations were made on the basis of both dry weight and milligrams of nitrogen. Since the results by the two procedures were comparable, only the Qo_2 values are presented.

Results. An increase in oxygen consumption of both liver and kidney slices was produced by the addition of alcohol (Fig. 1). The maximal increase occurred at an alcohol concentration of about 100 micromolar (about 0.5% w/v). Then, the oxygen consumption rapidly and progressively decreased to half its value at an alcohol concentration of approximately 1000 micromolar. The depressing effect of alcohol on respiration occurs more quickly and seems to be slightly greater for liver than kidney slices.

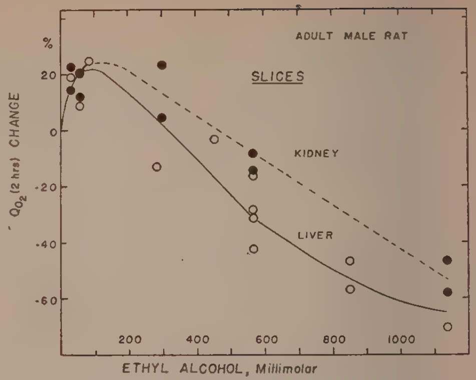

FIG. 1. Change in oxygen consumption of adult male rat tissues by various concentrations of ethyl alcohol.

Discussion. These results emphasize the importance and necessity of consideration of the concentration of alcohol in determining its influence on intermediary metabolic reactions.

Brain slices(3,4) also respond to the addition of ethyl alcohol in a manner similar to that of liver and kidney slices. The initial increase, however, is much smaller. This difference may be due to the fact that sufficiently small concentrations of alcohol were not used in the brain studies. The depressing effect of alcohol on brain tissues(4) was comparable to that obtained with kidney slices.

Summary. The addition of ethyl alcohol to liver and kidney slices from adult male rats produces a stimulation in oxygen consumption followed by a progressive inhibition as the concentration of the ethyl alcohol is increased.

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Received July 17, 1951. P.S.E.B.M., 1951, v77.

Hypervitaminosis A. An Adjunct to Present Methods of Vitamin A Identification.* (18937)

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The synthesis of vit. A and closely related compounds constitutes a field of investigation engaged in by numerous organic chemists. Once a new product has been prepared, its chemical structure elucidated and its spectrophotometric characteristics established, there remains the question of its biologic potency, for which no rapid methods of determination are available. The biologic assay procedure as given by USP XIV, including a depletion and assay period, requires a minimum of 46 days or a maximum of 73 days for completion. (U.S. Pharmacopoea XIV, p. 790, 1950).

This report offers a method for identifying as well as roughly evaluating the degree of biologic potency in terms of vit. A activity which is—(1) more rapid; (2) more specific; (3) requires no preliminary depletion period or use of specialized diets. This method depends upon the response of the skeletal structures of the young growing animal to excessive intake of vit. A. This response is specific in the sense that no other vitamin or any compound not closely related structurally to vitamin A gives a similar picture. The syndrome of hypervitaminosis A, with its emphasis upon changes in skeletal structures, has been repeatedly reaffirmed ever since its recognition by Collazo and his associates. Its characterization as an acceleration of normal growth sequences in long bones, first suggested by Wolbach and Bessey(1) was fully described by Wolbach(2). This response is readily recognized histologically in suitably prepared material when high dosage levels are maintained even for short periods of time. If one uses a weanling rat (the test animal that is most convenient and responds with great uniformity at high dosage levels) a tentative diagnosis can be made within 5-6 days, to be confirmed by histological findings in another 10 days, if the material under investigation has a potency in the range of vitamin A. If its potency is less, the experiment may consume a period of 3-4 weeks. If no potency is demonstrable on the basis of the histological picture at that time one can, with safety, state that the product compares so unfavorably with vitamin A that its potency is negligible.

The method has limitations in that it can be used only: (1) where high concentrations of a substance are available, and (2) where histological technics for bone study purposes are available and the interpretation can be made by one familiar with normal growth of rat bone.

Method. Weanling rats (their breed is immaterial) 21 days of age, preferably 35-45 g in weight, are maintained on an adequate stock diet and fed amounts of the preparation at dosage levels which are the equivalent of .3 mg of vit. A alcohol per gram of body weight per day for a period of 5 to 6 days or longer, or until fractures are grossly evident. If there is enough of the preparation available, 3 rats should be selected, one to kill at the end of 5 or 6 days, or before if fractures are evident, one to continue for 14 days on this same regime, and one for 3 weeks. With potent preparations, animals will probably not survive the longer periods. At the same time a minimum of 2 animals of the same weight and age should be maintained on a dosage of .3 mg per g of body weight per day of vit. A in a preparation known to produce fractures within 5 to 6 days at this level. These should be killed when fractures become evident and will serve as control material against which the response of the product that is being tested may be compared. Initial weights of animals should be recorded, as well

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* This investigation was supported by research grants from the Nutrition Foundation and the Williams-Waterman Fund for the Combat of Dietary Diseases.

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as daily changes in weight. Daily food intakes should also be charted.

If the preparation compares favorably in potency with vit. A, the syndrome begins to manifest itself within 24 hours by loss of activity on the part of the rat, decreased food intake, failure to gain normally or to gain at all, and usually a slight reddish discharge around the eyes. These signs should be noted, also any tenderness on pressure over the long bones. Within 5 to 6 days the animal will show gait difficulties, favoring either one or the other hind leg, or a front paw. With further dosing, fractures will occur and may involve any or all long bones. The most common early site of fracture in our experience has been the tibia, the next most common the femur. With vit. A—and this applies to both the naturally occurring product and the synthetic vit. A—recognizable histologic changes will occur after 2 to 3 doses at this level of intake.

If the supply of material is limited and only one animal can be used, and if there are no evidences of fractures at the end of the 5 to 6 day period, it is advisable to carry the one animal through the 3-week period before killing. If the preparation compares at all favorably with vit. A, it will show results after this interval.

Animals when sacrificed are carefully examined, special attention being paid to the long bones and the structures surrounding them. Hemorrhages into muscles and connective tissue structures are usually evident around fracture sites. The bones, after 18-24 hours' fixation in Zenkers, with subsequent washing, are decalcified in a 5% aqueous solution of nitric acid, embedded in celloidin, and stained with hematoxylin-eosin.

The changes to be looked for as results of vit. A activity have been described(2). Preferred for this purpose are longitudinal sections through the knee joint, cut in the sagittal plane, including one-half to two-thirds of the length of tibia and femur. The changes produced by a potent preparation of vit. A are wholly the result of acceleration of normal growth sequences and include the effects of an increased rate of maturation and vascular penetration of epiphyseal cartilage cells and increased activities involved in remodelling processes of bone, including resorption of bone attended by increased numbers of osteoclasts and new deposition of bone in conformity with the normal growth pattern.

In a group of rats at any age before completion of growth there may be considerable variation in thickness of the cartilage of the proximal tibia and distal femur epiphyses. In hypervitaminosis A the epiphyseal cartilage in growing rats becomes thinner than the extreme of the normal variation. The reduction involves all layers of the epiphyseal cartilage because all sequences, with the exception of proliferation, are accelerated. If the account of the normal rat epiphyseal cartilage, as described by Dodds and Cameron(3) is followed, the zone of "reserve cells", their Zone 1 (the layer nearest the epiphysis) remains unchanged. We have seen no indication that this layer gives rise to new cells to be incorporated into Zone 2, the zone of flattened cells and cell multiplication. Zones 2, 3 (the zone of cell growth) and 4 (the zone of fully grown cells, with adjacent calcified cartilage matrix) all became much narrowed as a result of rapid growth and maturation of the cells composing these layers (Fig. 1). In young guinea pigs administered excessive amounts of vit. A, these sequences result in complete closure of the epiphyses of the long bones in a period of 10 to 12 days.

Knowledge of the normal is required for the appraisal of epiphyseal cartilage changes in the rat resulting from hypervitaminosis A, inasmuch as complete consumption of the tibial proximal epiphyseal cartilage does not occur. It is to be recalled that normally in the rat the epiphyseal cartilages of the distal end of the femur and proximal end of the tibia persist usually throughout the life of the rat. Not infrequently in the 5- to 7-day period of excessive vit. A administration to rats there is little apparent diminution in width of the epiphyseal cartilage since increase in size (growth) of the cartilage cells has resulted. The number of cells per column, however, is reduced at the expense of Zone 2, *i.e.*, the zone of flattened cells and cell multiplication.

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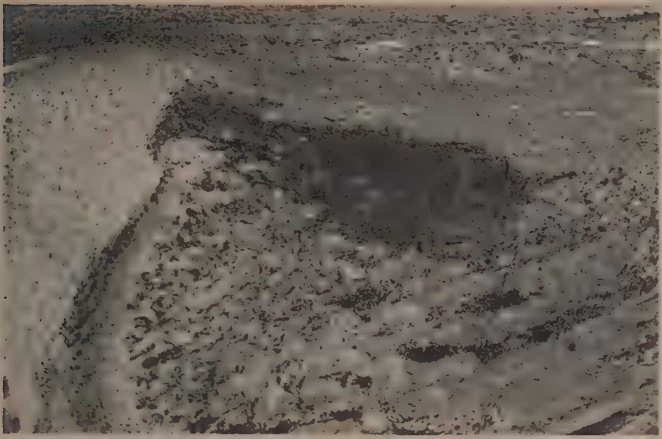

FIG. 1. Proximal end of femur, anterior aspect. Rat, six days' administration of natural vitamin A concentrate, 1000 I.U. per g wt. Note hemorrhage, the result of rapid remodelling. 80 diameters.

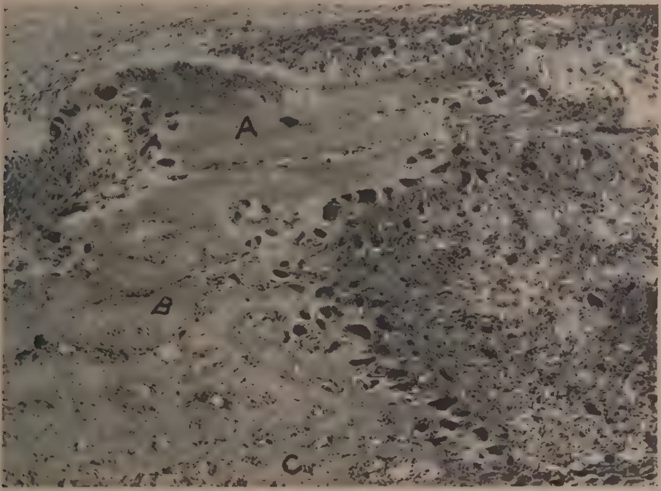

FIG. 2. Shaft of tibia. An oblique section through the tuberosity. Eight days' administration of synthetic vitamin A acetate. To show—(A) remnant of unresorbed bone; (B) region of new bone deposition. Note the extraordinary number of osteoclasts. 80 diameters.

The most marked reduction of width of the cartilage is at the periphery of the epiphyseal plate where there may be complete consumption of Zones 2, 3 and 4.

The changes in trabecular and cortical bone produced by excessive vit. A administration are so distinctive that less experience is required for their recognition.

The concavity of the shafts of bones—the tibia, for example—immediately adjacent to the epiphysis is maintained by resorption of the trabeculae as they merge into the cortical bone. In hypervitaminosis A a large excess of osteoclasts over the normal is apparent. The rapidity of resorption is such as to result in weakening of the periosteal attachments, hence small hemorrhages are often found in this region (Fig. 1). Farther

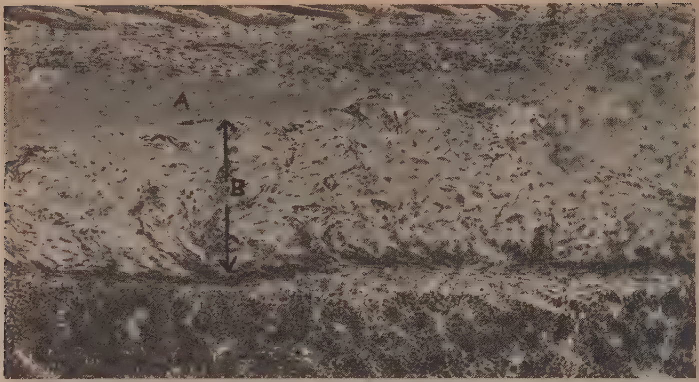

FIG. 3. Shaft of tibia, upper third. Three days' administration at 1000 I.U. per g wt, followed by 3 days at 1500 I.U. per g wt. Synthetic vitamin A acetate. Longitudinal section (A) and (B) to show the remains of the cortical bone still undergoing resorption; (C) new bone on the interior of the shaft. 113 diameters.

removed from the epiphyses where compact cortical bone must be removed, there is extensive osteoclasis on the external surface of the shaft and voluminous deposition of new bone on the interior of the shaft in the same regions (Fig. 2 and 3). This resorption and new deposition of bone follows precisely the normal growth pattern; only the rate of the processes has been changed. This has been determined by comparisons of normal femurs and tibio-fibular complexes with the corresponding hypervitaminosis A bones in serial sections.

If a compound compares favorably with vit. A in potency, fractures at the dosage levels used may occur in 5 or 6 days. They occur at sites where remodelling in normal growth is most active in regions subject to mechanical stress. Loss of previously formed cortical bone and lack of firmness of the newly formed bone explains the fractures. The lack of firmness of the newly formed bone is the result of incomplete calcification, expressive of the lag in calcification of bone matrix in normal bone growth as illustrated by the noncalcified matrix or osteoid present in appositional bone formation.

In applying this method for identification, the duration of the test period in the absence of fractures should be extended to 10 to 20 days, by which time the histological study of the bones should give the answer.

The advantages of the procedure may be summarized as follows:

(1) It is specific in the sense that no other substance administered under these conditions gives the response elicited by vit. A in skeletal structures. (2) No preliminary depletion period is necessary. This greatly shortens the time required for testing. (3) No special diets are required. (4) Numbers of animals in any one group may be very small—in fact, one animal is enough for a definitive answer. (5) Differences in potencies of vit. A preparations can be brought out by this method. For example, equivalent dosages of a synthetic crystalline vit. A acetate and of a concentrate of natural vit. A produced identical results with fractures by the sixth day.

Vit. A methyl ether in equivalent doses proved less potent than vit. A acetate or vit. A alcohol. There were no fractures during a 6-day period either with the crystalline product or with a concentrated solution supplied us by Dr. E. L. Sevringhaus, Director of Clinical Research of Hoffmann-LaRoche, Inc.[†] Results, *i.e.,* fractures comparable to those produced by the crystalline synthetic vit. A acetate or the concentrate of the natural vit. A were produced in one rat after

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[†] We are indebted to Hoffmann-LaRoche, Inc., for the synthetic vit. A products used in these experiments.

23 days of administration. Four rats sacrificed after a 6-day period showed easily recognized histologic changes typical of excessive vit. A intake.

Vit. A phenyl ether proved to be without effect in rats run for periods of 6 to 11 days.

The limitations of the method are as follows: (1) It can be applied only when large dosages are used. The syndrome is not produced by small amounts of pure products or by oils low in potency. (2) In the absence of fractures, adequate histologic sections of bony structures (knee joint) must be used for diagnostic purposes. (3) The histology must be interpreted by one familiar with the typical response.

Received July 18, 1951. P.S.E.B.M., 1951, v77.

Isolation of an Androgenic Compound from the Adrenal Venous Blood of Cows.* (18938)

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Although it has been thought that the adrenal gland may be capable of secreting androgenic compounds, the evidence presented has been inconclusive and other alternatives have seemed equally possible. Sayers(1) has suggested that such androgenic material may be the result of subsequent metabolism of the C_{21} steroids secreted by the adrenal cortex.

Besides 17-hydroxycorticosterone and corticosterone the presence of small quantities of other compounds in the adrenal effluent was indicated by previous work(2,3). The nature of these substances, however, was not determined.

In an attempt to obtain larger quantities of these compounds for identification, a special method was devised for the cannulation of the adrenal sinus of the cow. This has permitted the collection of large volumes of adrenal venous blood from cows stimulated with ACTH. At least 2 other compounds have been separated in addition to 17-hydroxycorticosterone(4). One of these fractions has now been demonstrated to have androgenic activity.

Experimental. Twenty liters of adrenal venous blood were collected from the adrenal sinuses of 3 cows which received an average of 600 mg ACTH intravenously during the blood collection. The animals also received intravenous infusions of heparin prior to the actual collection of blood and 5% glucose in saline during the 2-4 hour experiments. The blood was laked with an equal volume of H_2O and extracted with chloroform. The chloroform-extracted lipids were first partitioned between 70% ethanol and hexane and then between 30% methanol and hexane. The ketonic fraction of the 30% methanol-soluble lipids was acetylated and chromatographed on neutral aluminum oxide (Harshaw). Elution from the column was carried out with hexane-benzene, benzene, benzene-ether, ether-chloroform, and chloroform.

A fraction was eluted by the benzene-ether mixtures. This had an absorption maximum in the ultraviolet at 240 mμ and gave a positive Zimmerman reaction with maximum ab-

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* This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service.

† Public Health Service postdoctoral fellow, 1950-51.

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TABLE I. Determination of Androgenic Activity by Chick Comb Inunction with Androstadiene, and Fraction Obtained from Adrenal Venous Blood.

Group	No. chicks	Treatment	Total dose, γ	Body wt (Bwt)		Comb wt				Testes wt		
				Init.	Final	mg	% increase	mg % Bwt	% increase	mg	% diff.	mg % Bwt
1	10	Ether oil	0	40	86	48	—	56	—	29	—	33
2	10	Androsterone	15	39	82	163	230	199	238	24	–17	29
3	10	Adrenal blood	1.6	40	70	64	33	91	63	20	–31	28
4	10	” ”	16	40	78	90	88	115	105	22	–24	29

sorption at 520 mμ(5). The infrared spectrum indicated an α, β-unsaturated ketone structure and the presence of hydroxyl groups. Chromatography of the semicarbazones formed from this fraction suggests the presence of two compounds.

This fraction was assayed for androgenic activity by a modification of the chick comb growth method(6). Three-day-old White Leghorn cockerels were sorted as to weight into groups of 10 chicks per group. The steroids and extracts to be tested were dissolved in a mixture of 20% corn oil and 80% diethyl ether. Each chick received daily for 10 days, 0.02 ml of the respective solutions by application to the comb with a micropipette. The chicks were sacrificed 24 hours after the last inunction. Body, comb, and testes weights were determined, and the latter fixed in Bouin's fluid for histological evaluation. The results are indicated in Table I. It is evident that this fraction from adrenal venous blood was androgenic in nature.

A similar fractionation was carried out on peripheral blood from the same cows. No material showing the characteristics of the adrenal blood fraction was found.

Discussion. Masculinizing tumors of the adrenal gland associated with the excretion of large quantities of androgenic 17-ketosteroids have suggested that the adrenal gland might produce such compounds. These glands are functioning abnormally however, and cannot be used as an indication of normal function. Signs of masculinization have also followed ACTH therapy. Finally isolation of an androgenic 17-ketosteroid, adrenosterone, from adrenal tissue has been taken as evidence in favor of the secretion of such compounds by the normal gland.

Other workers, however, have believed that conversion of a C_{21} compound to an androgenic substance by peripheral tissues might account for the masculinizing effects seen after stimulation of the adrenal. The amounts of androgenic material found in adrenal tissue, even when the gland is largely made up of a masculinizing tumor, are very small. Since large amounts of 17-ketosteroids are excreted, it has been thought that such compounds as 17-hydroxycorticosterone and cortisone might be metabolized to these excretion products in other tissues of the body. The androgenic compounds isolated from adrenal tissue have likewise been considered to be possible intermediates in the production of the C_{21} steroids.

A comparison of the well known marked increase in urinary 17-ketosteroids following ACTH administration with the small conversion of exogenously administered C_{21} adrenal steroids to urinary 17-ketosteroids supports the hypothesis that the adrenal gland is able to secrete steroid compounds having the 17-ketone structure. This hypothesis is confirmed by the observation that 17-ketosteroids are present in the adrenal venous blood of the cow following stimulation of the adrenal cortex by ACTH. The same fraction has androgenic activity. It seems probable, therefore, that the androgenic effects and excretion of 17-ketosteroids which are seen after stimulation of the normal adrenal with ACTH are directly due to compounds secreted from the adrenal glands.

Summary. A fraction isolated from adrenal venous blood of cows has been demonstrated

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to have androgenic activity and to contain a compound giving the reaction for 17-ketosteroids. Its exact chemical structure has not been established due to the small quantity of the substance available for analysis.

Received July 18, 1951. P.S.E.B.M., 1951. v77.

Action of Calcium on Aerobic and Anaerobic Phosphorylation in Malignant and Certain Normal Tissues. (18939)

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In a previous communication(1) measurements of phosphorus uptake by homogenates of liver, brain, and tumor, and by the particulate and supernate fractions obtained by centrifuging such homogenates, were reported. The measurements were made under both aerobic and anaerobic conditions; the results indicated that phosphorylation could be effected by two different systems. In one system, associated chiefly with the particles and more prominent in liver than in tumor homogenates, phosphorus uptake was coupled with aerobic oxidation of succinate or other substrates of the tricarboxylic acid cycle; in the other system, associated chiefly with the supernate and quantitatively more important for the tumor homogenates, phosphorus uptake was coupled with anaerobic, glycolytic breakdown of hexose diphosphate.

Phosphorylation by the aerobic particulate system was, in confirmation of previous workers(2,3), blocked by 10^{-6} to 10^{-5} M 4,6-dinitro-o-cresol (DNC); in contrast, the phosphorylation by the glycolytic mechanism was not impaired by 10^{-4} to 10^{-3} M DNC. The present experiments deal with the sensitivity of these two phosphorylating systems to calcium ion.

Results. The effects of calcium upon phosphorus uptake by homogenates, particles, and supernate were measured with hexose diphosphate and pyruvate as substrate under exactly the same conditions as those described in the previous paper(1). Under these conditions part of the phosphorylation by the homogenate is associated with aerobic oxidation of pyruvate (chiefly by the particles) and part with glycolysis of hexose diphosphate (chiefly by supernate).

Calcium ion reduced the phosphorus uptake by particles from liver from 7.3 to –0.4 micromoles and by particles from brain from 8.5 to 1.5; phosphorus uptake by particles from the tumor, already low in the controls, was not further reduced by 0.0028 N calcium ion (Table I). The effect of calcium on phosphorus uptake by liver and brain particles under these conditions was a specific one, as oxygen consumption and lactate production were not significantly impaired at concentrations up to 0.0028 N Ca^{++}.

The phosphorus uptake by the supernate fraction from liver, brain, or tumor was not inhibited by calcium at concentrations up to 0.0028 N (Table I); there was also no impairment of oxygen consumption or lactate formation by these supernate fractions. The discrepancy between the phosphorus uptake of the tumor homogenate and the sum of the phosphorus uptakes of the washed tumor particles and supernate was found, as in the previous paper, to be due to the fact that when the tumor particles were shaken with a volume of buffered KCl equal to that of the original homogenate and recentrifuged, the wash supernate was found to contain an unexpectedly large amount of the glycolytic phosphorylating mechanism previously found in the original supernate. Since it appeared improbable that such large amounts of this

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TABLE I. Effect of Calcium on Phosphorus Uptake, Lactic Acid Production, and Oxygen Consumption by Homogenates of Rat Tumors, Brains, and Livers, and by the Particulate and Supernatant Fractions Derived Therefrom by Centrifuging at 20,000 g for 30 Min; the Particles Were Washed Once with Buffered KCl, Recentrifuged and Resuspended in Buffered KCl. Incubation time, 40 min at 25°C. Substrate, hexose diphosphate and pyruvate. Gas phase, air. Each experiment is based on material derived from 250 mg original tissue; for other experimental details see (1). All results are expressed in μMols.

		Normal rat liver			Normal rat brain			Walker tumor 256		
Preparation	Conc. of $CaCl_2$, equivalents per l	P uptake	Lactate formed	O_2 uptake	P uptake	Lactate formed	O_2 uptake	P uptake	Lactate formed	O_2 uptake
Homogenate	0	17.6	3.8	10.5	18.7	6.2	8.1	13.2	10.2	2.2
	0	17.7	3.3	10.3	19.3	6.4	8.2	13.5	10.5	2.7
	.00035	17	4.2	11.2	17.2	5.3	8.1	12.5	10.8	2.8
	.0007	16	3.7	13	15.8	6.9	8.5	10.2	11.1	2.4
	.0014	7.9	4	14.4	12	6.2	7.8	9.6	10.7	2.6
	.0028	5.2	4.4	13.6	9.8	6.6	7.8	8.8	10.7	2.7
Particles	0	7.3	1.1	7.3	8.5	3	4.8	2	3.7	1.7
	0	7.3	.7	8.1	8	3.2	4.9	1.9	3.7	1.4
	.00035	7.1	.6	8.9	5.6	3	4.6	2.3	3.4	1.5
	.0007	2.1	.8	9.6	5.6	3	4.7	1.7	4.1	1.4
	.0014	1.5	.6	7.1	2.9	3.4	4.6	2	3.6	1.5
	.0028	−.4	.6	5.7	1.5	2.5	4	1.9	3.3	1.2
Supernate	0	8.1	6.2	2.6	9.8	6.4	2.7	7.3	9.1	2
	0	8.3	5.8	2.4	9.8	6.3	2.6	7.5	8.4	2.2
	.00035	8.7	5	2.7	9.7	6.3	2.6	6.8	8	2
	.0007	8.7	7.5	3.4	9.6	6.8	2.7	7	8.2	2.5
	.0014	8.5	7.8	3.9	9.8	7.1	2.6	7.6	8	2.2
	.0028	8.7	7.8	3.8	9.9	8	2.6	6.8	8	2.2

material could be obtained by merely washing the particulate components, but must be derived from the particles themselves, either by diffusion out of or by partial disintegration of the particles, this matter has been made the subject of a special investigation, the results of which will be reported in a forthcoming publication.

The effects of calcium upon the phosphorus uptake which is coupled with aerobic disposal of α-ketoglutarate were also determined* (Table II). The phosphorus uptake of washed liver particles was blocked completely by about 0.001 N calcium; under these test conditions, in contrast to those obtained with the different test conditions of Table I, there was a substantial inhibition by calcium of oxygen uptake.†

Inhibition of phosphorus uptake by 0.00035 N Ca^{++} was prevented by 0.0014 N citrate (Table II); that by 0.0007 N Ca^{++} was not completely overcome by 0.0028 N citrate.

Repeated attempts to obtain washed particles from a tumor homogenate that would give a phosphorus uptake with α-ketoglutarate as substrate, using the system employed in the experiments with liver particles reported in Table II, have been uniformly unsuccessful.

In contradistinction to calcium, magnesium appeared to exert no effect on the phosphorus uptake and oxygen consumption by the washed liver particles. Magnesium chloride is present in the system employed at a concentration over 10 times that at which calcium completely blocked oxidative phosphorylation. In an experiment in which the magnesium content was varied over a range from 0.0132 M to 0.0016 M the phosphorus uptake and oxygen consumption remained unchanged.

A review of the results reported in this and the preceding paper brings out the fact that while oxidative phosphorylation appeared

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* The medium employed in these experiments (Table II) was the same as that employed in those reported in Table I and in the preceding paper with the exception that potassium bicarbonate, nicotinamide, diphosphopyridine nucleotide, and hexose diphosphate were omitted.

† Lehninger(4) has noted that calcium chloride, when employed at a concentration of 0.01 N, inhibited aerobic phosphorylation by washed liver particles, but he does not appear to have reported the effect of the calcium ion on phosphorylation in the lower critical ranges which have been dealt with here.

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TABLE II. Effect of $CaCl_2$ and Sodium Citrate, Singly and in Combination, on Phosphorus and Oxygen Uptakes by Rat Liver Particles Derived from a Rat Liver Homogenate by Centrifuging at 2,000 g for 15 Min; Particles Were Washed Twice with Buffered KCl Solution and Resuspended in Buffered KCl. In each experiment 250 mg wet wt of washed and centrifuged particulate system was employed. Substrate, α-ketoglutarate. Gas phase: air. Incubation time, 40 min at 25°C; results expressed in μMols.

Conc. of $CaCl_2$ equivalents per l	Conc. of sodium citrate equivalents per l	Experiment No. 1		Experiment No. 2	
		P uptake	O_2 uptake	P uptake	O_2 uptake
0	0	16	5.6	17.2	5.7
0	0	15.9	5.2	17.2	4.9
.00018	0	15.9	5		
.00035	0	5.7	2.2	5.8	2.7
.0007	0	1.3	1.9	1.4	1.6
.0014	0	−2.6	.9		
.0028	0	−2.6	1		
0	.00035	16	4.8		
.00035	.00035	4.3	2		
0	.0007	16	4.9		
.00035	.0007	8.5	2.6		
.0007	.0007	1.8	1.2		
.0014	.0007	−5.4	.5		
0	.0014	16	5.3		
.00035	.0014	15.5	4.5	9.4	3.1
.0007	.0014	4.4	.9		
.0014	.0014	−4.4	.8		
0	.0028			17.2	5
.0007	.0028			3.4	2.1

to be always dependent on the presence of particles and the water-soluble phosphorylating mechanism that appears in the supernate is invariably anaerobic in type, there is some evidence in the case of tumor and brain particles but not liver particles that an anaerobic mechanism resistant to DNC, calcium chloride, and anaerobiosis is either associated with or actually contained within some particulate component. This problem will be made the subject of a future investigation.

Summary. Phosphorus uptake and oxygen consumption by particles from rat liver, with α-ketoglutarate as substrate, are almost completely inhibited by 0.0007 N Ca^{++} and completely inhibited by 0.0014 N Ca^{++}; this effect may be a specific one upon phosphorus uptake or transfer, as the oxygen uptake with pyruvate and hexose diphosphate as substrate is not markedly impaired at the same Ca^{++} concentrations. Citrate used in concentrations equivalent to the calcium had no protective effect against the Ca^{++} inhibition, but larger ratios of citrate to calcium afforded partial or complete protection. Attempts to obtain oxidative phosphorylation with washed particles obtained from Walker 256 rat tumor homogenates, using α-ketoglutarate as substrate, were unsuccessful. Wide variations in concentration of magnesium appeared to exert no effect on the phosphorus uptake and oxygen consumption of washed rat liver particles.

When homogenates, particles, and supernates of rat liver, rat brain, and the Walker rat tumor 256 were subjected to the action of calcium with hexose diphosphate and pyruvate as substrate, the maximal inhibiting effect on the phosphorus uptake of homogenates and particles was noted with liver, an intermediate effect was obtained with brain, and a minimal effect was found with tumor. In all cases the phosphorus uptake, lactate production, and oxygen uptake of supernates were unaffected by calcium chloride at concentrations up to 0.0028 N.

The authors wish to express their appreciation to Dr. M. E. Krahl, of Washington University, for his advice in planning and interpreting these experiments.

The authors also wish to express their indebtedness to Dr. Paul N. Harris, who has provided them with normal rat brain and liver tissues and an ample

supply of pathologically controlled, Walker 256 rat tumors at suitable stages of development.

The authors also desire to express their indebtedness to Miss C. Patricia Walters for technical assistance without which these experiments could not have been conducted.

Received July 18, 1951. P.S.E.B.M., 1951. v77.

The Effect of Unrelated Vaccines on the Localization of Paralysis in Mouse Encephalomyelitis. (18940)

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Recent studies of poliomyelitis suggest that the inoculation of unrelated prophylactic and therapeutic agents increases the risk of paralysis in the inoculated limb(1-4). The importance of the observation to immunization programs and its implications regarding the pathogenesis of poliomyelitis prompted us to seek an experimental counterpart so that the phenomenon might be studied in the laboratory. The results of mouse tests in which the Lansing strain of poliomyelitis virus was used were irregular but suggested that the injection of vaccine may hasten the onset of paralysis, as has recently been reported(5,6). It was thought that mouse encephalomyelitis might prove more suitable since it is a natural disease of mice and is in so many respects similar to poliomyelitis. The present report describes experiments in which the introduction of vaccines apparently did increase the frequency of paralysis in the inoculated extremity following the intracerebral injection of mouse encephalomyelitis virus.

Methods. The mice used in the present experiments were of the Albany Swiss and Standard strains. Groups of 20 to 60 animals, 3 to 5 weeks old, were assembled by stratified random sampling.

The prophylactic agents tested, fluid-medium pertussis vaccine(7), precipitated diphtheria-tetanus toxoid, precipitated diphtheria-tetanus toxoid with pertussis vaccine, and typhoid-paratyphoid vaccine, were prepared in this laboratory(8). The bacterial count of the pertussis vaccine and of the triple combined product was 20,000 million per ml; that of the typhoid-paratyphoid vaccine 1,500 million per ml. The vaccines and other test substances were injected subcutaneously in the left front leg in 2 doses of 0.1 ml amounts a week apart at varying intervals in relation to the virus inoculation. A front leg was chosen for the site of vaccine injection because the virus strain employed induces paralysis in the front legs less frequently than in the hind legs(9).

A TO strain of mouse encephalomyelitis (Theiler) virus, No. 4727, was used throughout. Stock 5% mouse brain suspensions were stored until use in a dry-ice cabinet. The virus was injected into the left cerebral hemisphere in doses of 0.03 ml of 10^{-2} or 10^{-5} dilutions. Control groups received virus or vaccine only or saline in place of vaccine followed by virus. The mice were observed daily for at least 21 days following the inoculation of the virus. Mice were destroyed as soon as paralysis appeared.

During the interval between the inoculation

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TABLE I. Local Reactions in Forelegs of Mice Following the Subcutaneous Injection of Various Vaccines.

			Degree of reaction*		
Vaccine	No. of mice	None	Slight to moderate	Severe	Impaired function
Pertussis	74	7	44	20	3
Pptd. diph.-tet. toxoid and pertussis	80	0	17	43	20
Pptd. diph.-tet. toxoid	78	10	63	5	0
Typhoid-paratyphoid	78	75	3	0	0
Pertussis bacilli of vaccine	108	0	42	63	3
Supernate of vaccine	104	102	2	0	0
Saline	76	75	1	0	0

* The mice with *slight to moderate* reactions were those with the least degree of swelling that could be palpated or seen. Those with *severe* reactions had more extensive and marked induration but no impairment of gait. Mice with *impaired function* were excluded from the tests.

of the mouse encephalomyelitis virus and the predicted appearance of paralysis the animals were carefully examined for local response to the vaccines. The degree of reaction was graded and all mice in which the local reaction might obscure or resemble paralysis were excluded from the experiments. As may be noted in Table I, inflammation was most frequently found following the injection of pertussis vaccine alone or in combination with precipitated diphtheria and tetanus toxoids. The reactions induced by the toxoids were less severe and typhoid-paratyphoid vaccine, saline, and the cell-free supernate from fluid pertussis vaccine caused little or no reaction.

Results. Of the prophylactic preparations tested, pertussis vaccine either alone or combined with toxoids yielded the most striking effects. Nine experiments involving more than 800 mice have been performed in which pertussis vaccine alone was used. In each case the frequency of paralysis in the inoculated limb was greater than in the control groups. The total score showed the frequency to be approximately 7 or more times greater than expected (Table II, Group 1). In each experiment the mean interval between the inoculation of virus and the onset of paralysis was shorter in the vaccine-treated groups. The differences were not statistically significant but the consistency in the 9 experiments proved to have a probability factor of 1:512. The frequency of paralysis *irrespective of location* was no greater in the vaccine-treated than in the control groups. The inoculation of saline did not have a measurable effect.

Experiments have been performed in which pertussis vaccine was given at various times in relation to the inoculation of virus. These also are summarized (Table II, Group 5). The localization of paralysis in the injected extremity was commonest in those mice which had received vaccine prior to or on the same day as the virus. When both doses of vaccine were delayed until several days after the virus, there was no effect.

Tests were made separately of the bacterial elements and the supernatant fluid of pertussis vaccine. The sedimented cells were washed once and resuspended in saline. The supernatant fluid was filtered through a Berkefeld candle. The results indicate that the effect is due to the cellular rather than the fluid component (Table II, Group 2).

The experiments in which diphtheria-tetanus toxoid and typhoid-paratyphoid vaccine were used are also summarized in Table II, Groups 3 and 4. The first yielded results similar to but less marked than those of pertussis vaccine. The inoculation of typhoid-paratyphoid vaccine had no effect.

It is interesting to note that the inoculation of vaccine alone has never been followed by paralysis although more than 600 mice so treated have been observed for 35 days or more, and the majority of these were presumably latently infected with mouse encephalomyelitis virus(10,11). Future experiments of

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TABLE II. Frequency of Foreleg Paralysis Following Injection of Vaccines.

Group No.	Vaccine injected	Time in relation to virus	No. of mice	Mean incubation period, days	Paralyzed No.	Paralyzed in injected foreleg*	
						No.	%
1	Pertussis		188	11.7	146	49	34
	Saline		193	12.9	151	7	5
	Pertussis	7 days before and on same day	224	12.7	155	52	33
	None		239	14.4	164	4	2
	Pertussis†		60	5	53	40	75
	None†		62	8.3	58	4	7
2	Pertussis	,,	100	13.9	63	22	35
	Cellular component		105	14.2	66	17	26
	Fluid component		104	14.5	69	1	1
	None		98	14.3	57	1	2
3	Pertussis	,,	138	11.7	106	34	32
	Pptd. diph.-tet. toxoid		142	13	111	19	17
	Pptd. diph.-tet. toxoid and pertussis		131	12.6	104	34	33
	Saline		144	12.9	118	6	5
4	Typhoid-paratyphoid	,,	78	13.8	64	1	2
	Saline		76	13.3	58	1	2
	None		79	13.7	69	2	3
5	Pertussis	14 and 7 days before	29		21	8	38
	,,	7 days before and on same day	28		24	10	42
	,,	4 days before and 3 days after	30		21	9	43
	,,	Same day and 7 days after	31		22	8	36
	,,	4 and 11 days after	30		23	2	9
	,,	7 days after (1 dose only)	29		21	3	14
	None		30		23	1	4

* Does not include mice paralyzed in both front legs.

† Virus inoculated intracerebrally as 0.03 ml of a 10^{-2} dilution of infected mouse brain; in all other experiments a 10^{-5} dilution was used.

this kind will be undertaken. The association of recent immunization and poliomyelitis in man could be due to the activation of a latent infection, the localization of active infection occurring during a critical period following vaccination, or to the introduction of virus with the vaccine. Nothing in the present experiments lends support to either the first or third hypothesis but neither should be dismissed because of mouse experiments.

Whether the paralysis-localizing and the inflammation-producing activities of the vaccine are identical remains to be proven. The present experiments suggest such an association.

Conclusions. Mice injected in a foreleg with pertussis vaccine alone or combined with precipitated diphtheria-tetanus toxoids were frequently paralyzed in the injected foreleg following infection with mouse encephalomyelitis virus. The frequency of paralysis in the inoculated extremity was 7 or more times greater than among the untreated controls.

Precipitated diphtheria-tetanus toxoid had less effect and typhoid-paratyphoid vaccine had none.

The injection of vaccines did not increase the frequency of paralysis, irrespective of location, but pertussis vaccine consistently shortened the period between the introduction of virus and the onset of paralysis.

Received July 19, 1951. P.S.E.B.M., 1951, v77.

Urinary Excretion of Vitamin B_{12}, Folic Acid, and Citrovorum Factor in Human Subjects on Various Diets.* (18941)

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Recent reports(1,2) have suggested that no appreciable vit. B_{12} activity is present in the urine of normal subjects. In the present study small but measurable amounts of vit. B_{12} have been found in the urines of 56 medical students who were maintained on various diets. Folic acid and citrovorum factor were also measured in the urines of these subjects to study the correlation, if any, in the excretion of these 3 vitamins.

Methods. Students in the medical school adopted various self-selected diets for a 3-day period and collected the 24-hour urine (under toluene) on the third day for metabolic studies in the biochemistry course. Aliquots of the urines were collected from the students, neutralized to pH 6.9, and stored in the frozen state until analyzed.

Vit. B_{12} was measured microbiologically by a modification of the method of Thompson *et al.*(3). Stock cultures of the assay organism, *L. leichmannii* 4797 were transferred weekly in a skim milk medium containing 0.5% Difco Bacto-yeast extract, 0.5% whole liver powder,† and 10 mγ vit. B_{12}‡ per 10 ml tube. The medium for growth of the inoculum was prepared by addition of 10 mγ vit. B_{12}, 5 mg liver concentrate 1-20§ and 5 mg Difco Bacto-yeast extract per 10 ml tube of assay medium. The tubes of media for stock and inocula cultures were autoclaved at 120°C for 15 minutes and stored in the refrigerator until used. In preparing the assay medium(3) the ascorbic acid was added after the medium had been neutralized to pH 7.0. The tests were incubated at 37°C for 65 to 72 hours and titrated with 0.1 *N* alkali. Many of the urine samples were assayed at 2 or 3 different dilutions with good duplication of values. Marked inhibition was observed in a number of samples when the urine was undiluted; however when diluted 2 to 4 times, the inhibition was greatly reduced or completely eliminated. Active material, other than vit. B_{12}, was measured in some of the urines after destroying vit. B_{12} by alkali treatment(4).

Folic acid activity (FA) was measured using *L. casei* 7469 with a medium modified from that suggested by Landy and Dicken(5) and synthetic pteroylglutamic acid (PGA)¶ as a standard. Citrovorum factor activity (CF) was assayed using *Leuconostoc citrovorum* 8081 with a medium similar to that of Sauberlich and Baumann(6) and synthetic CF¶ as a standard. All assays were done titrimetrically after 65- to 72-hour incubation. Fresh samples of synthetic CF were found to

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* This work was supported by grants from the Nutrition Foundation, Inc., The Roche Anniversary Foundation, the Williams-Waterman Fund of the Research Corporation and the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

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‡ Crystalline vit. B_{12} was generously furnished by Merck and Co., Inc., Rahway, N. J.

§ Liver concentrate 1-20 was generously supplied by the Wilson Laboratories, through the courtesy of Dr. Claire E. Graham.

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¶ Synthetic PGA and CF were kindly furnished by the Lederle Laboratories Division, American Cyanamid Co., through the courtesy of Drs. E. L. R. Stokstad and H. P. Broquist.

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TABLE I. Average Excretion of Total FA, CF, and Vit. B_{12} Activities in Urine of Human Subjects on Third Day of Dietary Regimen.

Diets	No. of subjects	Vit. B_{12} activity, mγ	FA activity, γ	CF activity, γ
Normal	14	31(16–55)	4.1(1.9– 6.6)	2.6(1.6–4.8)
Milk	7	37(11–70)	2.6(1.4– 4.7)	1.8(1.2–3)
Alkaline ash	6	28(6–54)	3.8(2.7– 6.4)	2.6(2.2–3.2)
Low protein	8	36(19–52)	3.3(2.3– 4.7)	2.8(2.1–4.1)
High "	7	33(13–52)	3.1(1.8– 5.2)	2.6(2.1–4)
" purine	8	43(15–79)	10.4(4 –25)	4.9(1.8–8.1)
Fasting	6	62(36–83)	3.9(2.7– 6.6)	2.8(2.2–3.8)

possess about 4% of the activity of PGA for *L. casei*. After 1 to 2 weeks the activity of the synthetic CF for *L. casei* was found to increase markedly when kept in dilute solution (30 γ/ml) in the refrigerator. The CF in the urines may therefore contribute variable amounts of activity to the FA values found with *L. casei.*

Results and discussion. The range and average daily excretion of vit. B_{12}, FA, and CF compounds in the subjects on the various diets are shown in Table I. The values given for vit. B_{12} may include other substances which are active for *L. leichmannii.* The average vit. B_{12} excretion by the fasting subjects was much higher than that found in any of the other groups. Assay of the urine after alkaline destruction of the vit. B_{12} showed that 4% (0 to 11%) of the total activity in the urine of the fasting subjects still remained. This was similar to the blank value 3% (0 to 8%) obtained for the urines of the subjects on the other diets with the exception of those on the high purine diets. For the high purine diets the average activity after alkaline treatment represented 32% of the total activity. This was presumably due to breakdown products of the nucleic acids found in the large amounts of liver and sweetbreads in these diets. After subtraction of these blanks, the average vit. B_{12} excretion values fell in the normal range. The true vit. B_{12} excretion value appeared to be about twice as high in the fasting groups as in any of the others. An increased excretion of other B vitamins has been observed during fasting in dog and man(7,8).

The controversial results in regard to the presence of the antipernicious anemia factor (vit. B_{12}) in the urine of human subjects (9,10) would be expected in view of the small amounts of vit. B_{12} excreted per day and the wide variation between different subjects. A concentrate of many liters of urine would be necessary to obtain sufficient vit. B_{12} for a reticulocyte response(10).

The changes in excretion of FA and CF compounds with diet appear to be similar (Table I). The excretion of FA and CF compounds by the subjects on the alkaline ash, low protein, high protein and fasting regimens was comparable to that found for those on the normal diets. A decreased excretion of FA and CF was evidenced in the subjects on the milk diet and a greatly increased excretion was found in the group on the high purine diet. The high values in the latter group were probably due to the same nucleic acid derivatives which were found to interfere in the vit. B_{12} assay. The excretion of FA and CF compounds was not increased during fasting, in contrast to that found for vit. B_{12}.

A part or all of the CF activity of the urine may be included in the FA values. In all individuals tested, the values for FA compounds were as high or higher than the corresponding values for CF compounds. This is summarized in the average figures (Table I) and suggests that other compounds in addition to CF may contribute to the total FA excretion.

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Summary. The average urinary excretion of vit. B_{12} in 14 subjects on normal diets was found to be 31 mγ per day. Similar values were obtained in 28 subjects maintained for 3 days on the following diets: milk, alkaline ash, low protein, and high protein. A somewhat higher value was found in 8 subjects on high purine diets, and the increase over normal was shown to be due to interfering compounds. On the third day of fasting, 6 subjects were found to excrete an average of 62 mγ vit. B_{12} per day.

The average CF excretion by the subjects on the above diets accounts for only part of the total FA compounds excreted, and appears to be correlated with the total FA values. Differences in vitamin B_{12} excretion resulting from dietary changes are not similar to those observed in CF and FA excretion.

Received July 23, 1951. P.S.E.B.M., 1951, v77.

Effect of Cortisone upon Chemical Composition of Allantoic Fluid of the Chick Embryo.* (18942)

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The studies herein presented were undertaken in a search for biochemical correlates of the striking changes in the growth, development, and survival of chick embryos treated with cortisone or certain other steroids(1-5).

Methods. Distilled water or dilutions of cortisone† in distilled water were inoculated into the yolk sac of fertile White Leghorn eggs on the fourth day of incubation. Allantoic fluids for chemical analysis were drawn from surviving embryos on the thirteenth day of incubation. This age was selected to avoid the excessive precipitate of urates in older embryos. The methods employed in the determination of sodium, potassium, chloride and sugar (reducing substance) levels are in regular use in this and in other laboratories(6).

Results. In Fig. 1 the chemical composition of the allantoic fluid from cortisone-treated chick embryos has been compared with the findings in controls inoculated with distilled water.

It is readily evident that cortisone treatment raised the mean value of the allantoic fluid sodium and lowered its potassium. This is a consistent finding irrespective of the amounts of hormone given. There is no dosage-effect relationship present despite the fact that the graphs suggest maximal increases in sodium in the 1.0 mg group with alterations in the same direction but of lesser magnitude with the 0.25, 0.5, 2.0, and 4.0 mg amounts. Statistically significant changes in the mean chloride and sugar (reducing substances) were

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* Aided by a grant from the Damon Runyon Memorial Fund for Cancer Research, Inc.

§ Damon Runyon Clinical Research Fellow.

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† Vehicle in which cortisone acetate is supplied by Merck and Co., Inc:

1.5% benzyl alcohol
0.9% sodium chloride
0.4% polyoxyethylene sorbitan monooleate
0.5% sodium carboxymethylcellulose
(distilled water q.s. ad 100%)

This suspension was diluted with water to permit the injection of 0.25 to 4.0 mg of cortisone in a constant volume of 0.2 ml.

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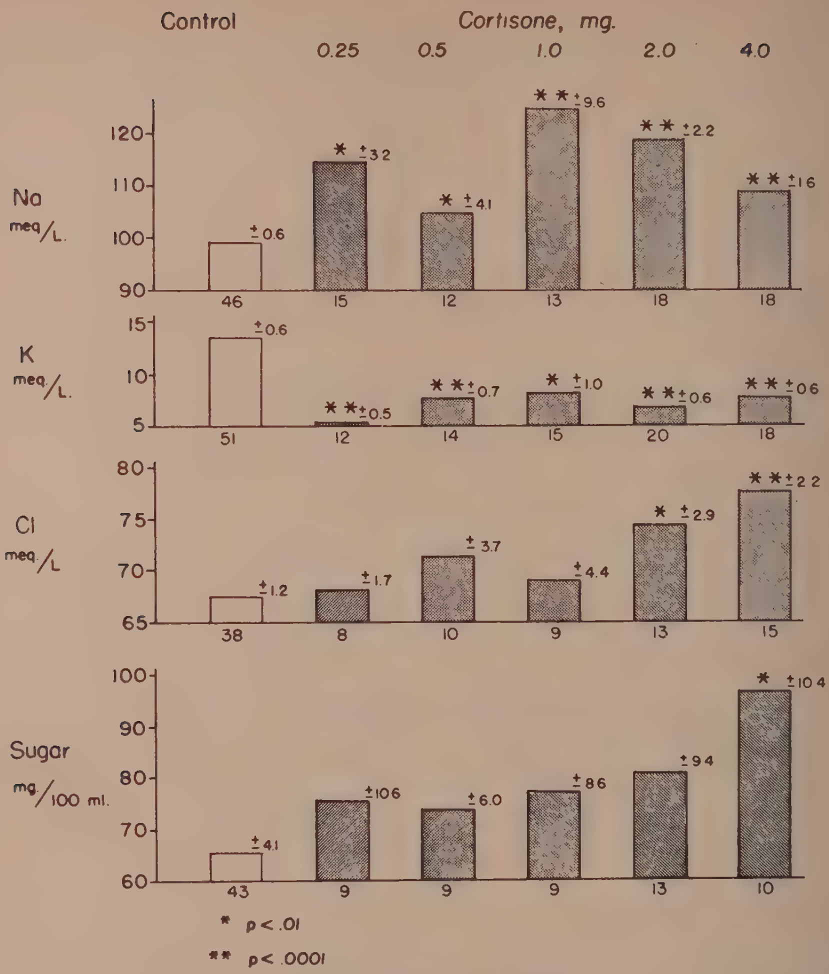

FIG. 1. Mean concentrations (± S.E.) of ions and reducing substances in allantoic fluids of 13-day-old control or cortisone-treated chick embryos. Injection of cortisone, 0.25 to 4.0 mg, into the yolk sac of 4-day-old fertile eggs increased the mean concentration of sodium and lowered the potassium. The chloride values rose in the 2.0 and 4.0 mg groups, while the reducing substances increased significantly in only the latter.

recorded only with the 2 larger and the largest dosages of cortisone, respectively. The findings are not attributable to changes in hydration, since the weights of the control and the treated eggs were indistinguishable at the end of the experiment. The opposing directions of the sodium and potassium alterations exclude the possibility that the observed changes merely indicate a decrease in the water of allantoic fluid.

Discussion. The allantoic fluid has been taken to represent the main excretory pool in the chick embryo(7,8). As such the effects of cortisone in these studies can be compared for purposes of discussion with those reported in the urine of human subjects receiving this steroid. The sodium and potassium effects are just the opposite of the changes in urine

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composition generally found to follow the initiation of cortisone therapy in human subjects(9), *i.e.,* the excretion of sodium into the allantoic fluid increased while the output of potassium decreased. These findings suggest that the chick embryo allantois is influenced by cortisone, directly or indirectly, but responds in a manner opposite to that characteristic of the human kidney. The increased concentration of chloride and of reducing substances in the allantoic fluid, on the other hand, is directionally similar to the changes induced by cortisone in humans.

It may be that exogenous cortisone interferes with the formation or function of the adrenal gland and thereby ultimately induces adrenal insufficiency. In such an event the observed cation and chloride changes would resemble those seen in human adrenal insufficiency(10). Since the unhatched chick lives and grows in a closed system, any increased output of sodium or decreased elimination of potassium must inevitably be accompanied by depletion of the former element from the body and excessive retention of the latter. Our data do not indicate whether the chemical changes mediate or merely accompany the retardation of growth, developmental anomalies, and decreased survival rates observed in cortisone-treated chick embryos.

Summary. 1. The observed rise in sodium and the decrease in potassium in the allantoic fluid of chick embryos treated with cortisone are the reverse of the reported changes in the urine of human subjects receiving this steroid, and resemble those seen in adrenal insufficiency. 2. The increased chloride and sugar levels in the fluid of the allantois following therapy with the higher dosages of cortisone resemble directionally the renal effects of cortisone in man.

Received July 23, 1951. P.S.E.B.M., 1951, v77.

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Inhibition of the Growth of Egg Cultivated Tumor Tissue by Extracts of *Cooperia pedunculata* Herb. (18943)

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During a survey of the effect on tumor tissue of various extracts of plants known to be toxic when ingested by animals, certain extracts of *Cooperia pedunculata** Herb. (Rain-lily) have proved to be inhibitory of tumor growth.

Acetone and acid aqueous extracts of the bulb of this plant when introduced into eggs bearing yolk sac implantations of mouse mammary carcinomas inhibited the growth of the tumor tissue almost entirely for the period of the test at dosage levels which did not appear to affect adversely the supporting chick embryos.

The plant *Zephyranthes atamasco* Herb. is known to be poisonous to live stock(1). The Rain-lily used in this study is a closely related species and probably has the same toxic properties.

No reports were found in the literature of the use of Rain-lily extracts in cancer chemotherapy.

Included in this report are results obtained when 4-aminopteroylglutamic acid (Aminopterin) was tested in the same way as the plant extracts. This compound is well known for its inhibitory effect on the growth of cancer and other tissues and the fact that this is readily demonstrated by injection into tumor bearing eggs is evidence of the validity of the approach.

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* Our appreciation is extended to Dr. W. V. Brown and Dr. G. S. Rabideau of the Department of Botany, University of Texas, for confirming our identification of this plant.

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Experimental. Rain-lily bulbs were collected in April. All of the procedures were carried out at as low a temperature as practical. The extracts were kept chilled or frozen until introduction into the test egg. Special care was taken to insure sterility in the solutions tested. The effective extracts were made as follows:

An *acetone extract* was obtained by immersing coarsely comminuted portions of fresh Rain-lily bulb in acetone in the ratio of 1 to 10 for a period of 24 hours. The acetone was then sterilized by passage through an ultrafine filter and evaporated at low temperature under reduced pressure. The resulting powder, weighing 282.4 mg per 10 ml of acetone was dissolved in sterile cottonseed oil.

An *acid (HCl) aqueous extract* (pH 6.6) was made by immersing in this acid solution 1 part of coarsely comminuted Rain-lily bulb to 10 parts liquid and allowing the mixture to stand 24 hours. At the end of this period the solution was filtered and sterilized in the same manner as the acetone extract. In a similar way *aqueous and alcoholic extracts* were prepared. One extract was made by pressing out the liquid from the plant tissue with a hydraulic press.

Aminopterin was dissolved in 1% Na_2CO_3 for injection. All the materials tested were introduced into the tumor-bearing eggs in the form of an emulsion consisting of 2 parts cottonseed oil and 1 part H_2O. Control eggs received the suspending fluid.

Tumor-bearing eggs were obtained by inoculating the yolk sacs of embryonated eggs on the 4th day of incubation with C_3H mouse mammary carcinoma tissue suspension. The details of the method have been previously described(2-4). The materials being tested were introduced into the tumor embryo system on the 12th day of incubation by injec-

TABLE I. Effect of Extracts of Rain-Lily Bulbs and Aminopterin on Growth of Tumor and Embryo of Tumor-Bearing Eggs. 24 hr test period.

Material tested	No. of eggs	No. of exp	Dosage	Survival control =100	Avg initial wt, g Tumor	Avg initial wt, g Embryo	Avg tumor wt end of test period, g Exp	Avg tumor wt end of test period, g Control	Avg embryo wt end of test period, g Exp	Avg embryo wt end of test period, g Control	Growth inhibition, % Tumor	Growth inhibition, % Embryo
Rain-lily acid-aqueous	112	4	.07 ml	100	.25	3.64	.29 ± .05	.45 ± .06	4.75 ± .13	4.73 ± .09	81	0
Rain-lily acetone	80	3	.5 mg	100	.27	3.62	.28 ± .01	.46 ± .03	4.80 ± .13	4.72 ± .12	94	–8*
Aminopterin	128	5	.1 mg	86	.24	3.30	.27 ± .03	.47 ± .04	4.30 ± .08	4.54 ± .09	87	19

* Indicates % growth acceleration.

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tion over the blood vessels of the chorioallantoic membrane and area vasculosa as described elsewhere(5-7). A test period of 24 hours was used during which time the yolk sac tumors nearly doubled in size. The effect on growth was evaluated on the basis of the increased size of embryo and tumor which occurred in the control eggs during the period of the experiment. A total of 320 tumor-bearing eggs involving 12 separate experiments was used in these tests.

Results. Table I records the results with 2 Rain-lily extracts and aminopterin. Aqueous and alcohol extracts of the plant were ineffective. Negative results were also obtained with a solution of liquid pressed from the plant bulbs at high pressure.

It will be noted in the table that the Rain-lily extracts gave about the same order of inhibition of tumor growth as did aminopterin but without the inhibition of the embryo growth which characterized the action of the latter compound.

Discussion. The chemical nature of the effective agent in these Rain-lily extracts will be explored. It is known that an alkaloid with fungicidal action is present in plants of this group. To obtain this material, dry powdered bulbs are extracted with 95% ethanol (8). In the present work ethanol extracts of fresh bulbs were ineffective in the tests on cancer growth. There is apparently a seasonal factor involved since extracts of bulbs collected in the Austin area in the late Spring after the plant was advanced in growth were ineffective.

Summary. Extracts of Rain-lily bulbs have been tested for their effect on the growth of tumor and embryo of tumor-bearing eggs. The results obtained with aminopterin in the same tests are included for comparison. Acid aqueous and acetone extracts of the plant bulb inhibited tumor growth 81% and 94%, respectively, without affecting the embryo. Aminopterin inhibited tumor growth 87% and embryo growth 19%.

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Received July 24, 1951. P.S.E.B.M., 1951, v77.

Electronmicrographs of Thin Sections of Molluscum Contagiosum.* (18944)

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The morphology of *Molluscum contagiosum* has been extensively studied under the light microscope. The usual description consists of cells of the epidermis which are altered by the presence of cytoplasmic inclusion bodies (the Henderson-Patterson molluscum body) appearing first in the Malpighian layer and reaching maturity in the lower stratum corneum. Van Rooyen(1) has demonstrated by microdissection that the mature globular inclusion body is filled with elementary bodies lying in a gelatinous matrix. Rake and Blank (2), in tracing the development of the inclusion body, maintained that there is first a

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* This work was done in part under an American Cancer Society Fellowship recommended by the Committee on Growth of the National Research Council and supported by research grants from the National Cancer Institute, National Institutes of Health, Public Health Service and the Fluid Research Fund, Yale University School of Medicine.

[†] Fellow, American Cancer Society.

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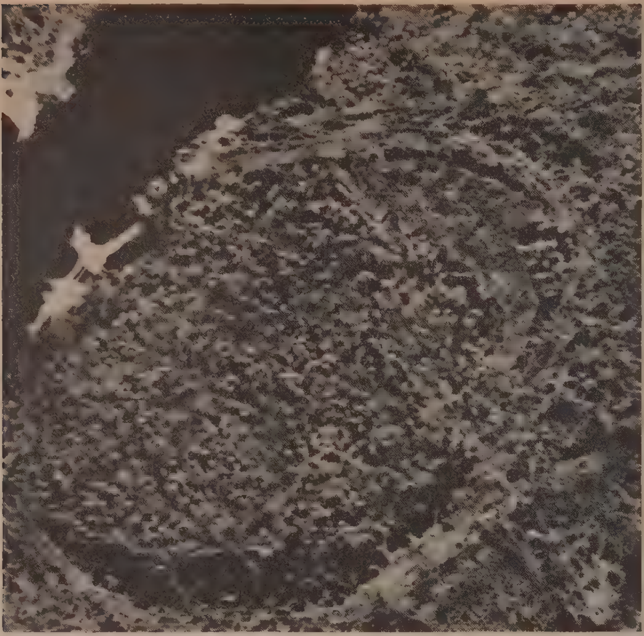

FIG. 1. Mature inclusion body in cell. The nucleus is shrunken and displaced to the side. × 4000.

general increase in cytoplasmic ribonucleic acid which is followed by the appearance of diffusely scattered islands of desoxyribonucleic acid. The latter enlarged to form the characteristic inclusion body within which these authors described septa containing ribonucleic acid. The elementary bodies from the supernatant portion of centrifuged material from the lesions of molluscum contagiosum have been found by electron microscopy to be brick-shaped(2-5) with average measurements of 330 mμ by 230 mμ(5). The present report extends the observations on the morphology and suggests possible stages in the development of the virus by the examination of thin sections of molluscum lesions with the electron microscope.

Methods. Four separate lesions of *Molluscum contagiosum* freshly removed with a curette were fixed in 4% buffered formalin for 2-3 hours. Pieces of tissue no larger than 1-2 mm on an edge were dehydrated in a graded series of alcohols and embedded in methacrylate (1 part methyl and 8 parts butyl), using 2-4 dichloro-benzoyl peroxide as a catalyst and incubation for 18-24 hours at 60°C(6). Sectioning was performed on a Spencer microtome adapted with a 1:20 wedge(7). The wedge and flywheel were counterbalanced and the microtome was driven by a motor(8). Sections from 0.05 to 0.2 μ thick were cut with a glass knife(9), mounted on collodion-covered grids, and, after removal of the methacrylate by xylol, were shadowed with palladium at an angle of approximately 1:7 before examination with an RCA electron microscope, model EMU. The illustrations

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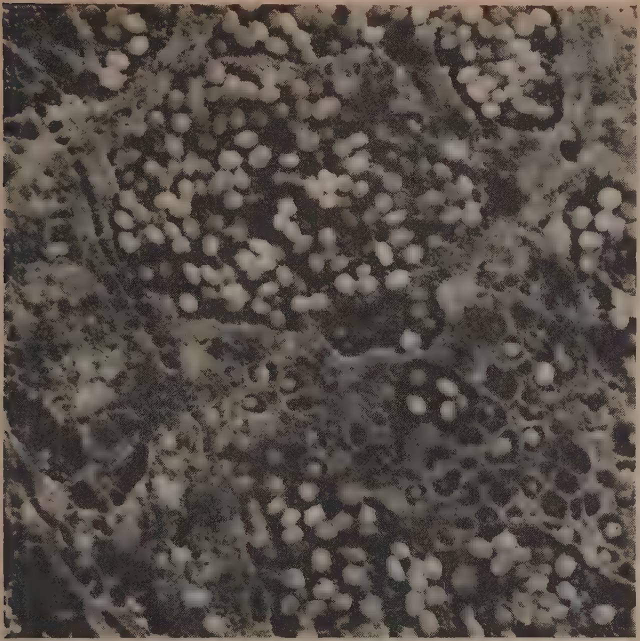

FIG. 2. Small portion of inclusion body. Locules containing discrete elementary bodies. Apparent segmentation within the matrix of septa with variation in form and size of the contained elementary bodies. × 11,000.

are enlargements from negatives made by contact with the original plates.

Results. In the prickle-cell layer of the epidermis, cells of the molluscum lesion contained within their cytoplasm varying numbers of elementary bodies which did not appear to be confined within a membrane and were fewer in number than in the mature inclusion bodies. In these cells the virus particles seemed to have no particular arrangement or localization, but were distributed at random within the cytoplasm. The shape of the elementary bodies, when only a few were present in the cell, often differed from that found in the mature inclusion bodies. Some were thinner in proportion to their length with typical measurements of 338 by 105 mμ, and at times were even lancet-shaped. The completely formed elementary bodies found in the more mature inclusions were generally smooth and more ovoid in outline than the early forms, with average measurements of 360 by 210 mμ. These are similar in appearance and size to elementary bodies obtained from suspensions of ground molluscum lesions.

The largest inclusion bodies are divided into compartments by septa with mature elementary bodies filling the locules between the septa (Fig. 1, 2). In some instances areas of the matrix composing these septa appear to be cut out into rounded or indented structures (Fig. 3). Some of these bodies are distinctly contoured and separated from the surrounding matrix by a more electron-translucent zone as seen in shadowed preparations (Fig. 3). All gradations in size and density can be found between these structures and the mature elementary bodies which would suggest that the latter may be formed by a segmentation and condensation of this matrix. In those early infected cells, where only a very few elementary bodies are present, no segmentation of matrix in the cytoplasm has been seen.

In several instances mature elementary bodies were found associated with smaller particles. In one preparation these particles closely resembled those pictured by Rake and Blank(2) but were somewhat smaller, measuring 76 x 61 mμ.

Further observations have been made on

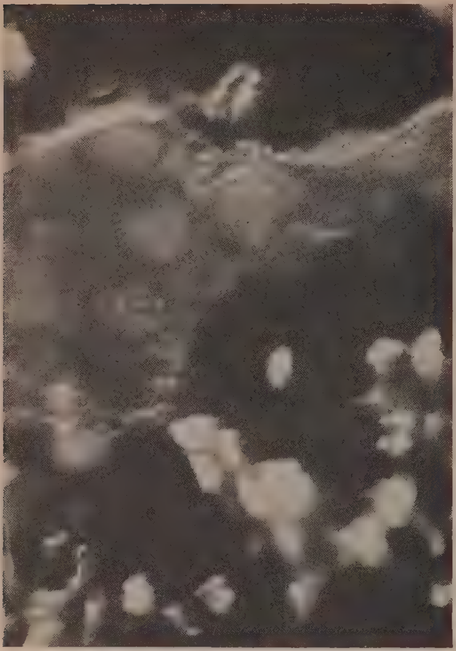

FIG. 3. Portion of septum with a few elementary bodies in adjacent locule. Segmentation of matrix of septum is evident. × 28,000.

the elementary body itself. In Fig. 4, there is a cluster of elementary bodies most of which have been cut twice leaving only a central segment which appears as a dense ring of material with an empty center. That this is a real observation appears certain from the many sections in which both intact and sectioned elementary bodies are seen. It is possible, then, that the elementary bodies of *Molluscum contagiosum,* at least in these instances, possess a formed cortex and a less dense interior.

Discussion. Thin sections of molluscum lesions examined with the electron microscope have suggested new concepts of the morphology and development of *Molluscum contagiosum.* The elementary bodies, in some instances, appear to have a dense wall and a less dense interior. The wall is of uniform density, of varied thickness and without demarcation between cortex and what might be adherent contents. Though the central portion, in some instances, appears empty, in others, apparently sectioned, no hollow is seen. It is possible then that there is a difference in the central portion of elementary bodies which may depend upon their stage of development. This is further suggested by the fact that hollow central areas are seen consistently in some groups of elementary bodies and not in others. It must be kept in mind, however, that it may also be possible for the bodies either to be cut or pushed to one side depending upon the sharpness of the knife and their orientation to the cutting edge.

Epidermal cells with only a few elementary bodies in their cytoplasm may be considered to represent an early phase of infection. There are all gradations from these to other cells that are almost completely filled by a well-defined inclusion body. When an abundant matrix separates the elementary bodies, it is often segmented into small areas of varying sizes and densities approaching that of the mature elementary body. In the larger inclusions, this matrix remains as thin septa yet within them the same process of segmentation and condensation can be observed.

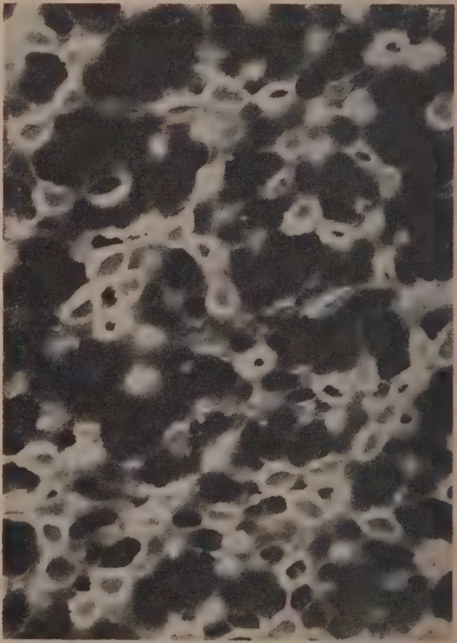

FIG. 4. Elementary bodies that have been sectioned. Note dense wall and apparently empty interior. × 34,500.

The septa are the thin hematoxylin-stained lines that can be seen crossing the inclusion body in hematoxylin-eosin preparations. Rake and Blank(2) have considered these septa to contain ribonucleic acid, basing this conclusion upon staining with pyronin-methyl green. Kurnick(10) has demonstrated that depolymerized desoxyribonucleic acid stains with pyronin similarly to ribonucleic acid. The locules between the septa, where the mature elementary bodies lie, contain desoxyribonucleic acid since these regions are stained by the Fuelgen method(2). It seems apparent, then, that the site of formation of the elementary bodies is in the material which is selectively stained with pyronin.

In the early infected cells, many of the elementary bodies are thinner than most of those found in the mature inclusions and in addition lancet shaped forms are seen. There is also an apparent absence of segmented matrix in the cytoplasm of these early infected cells which leaves the origin and fate of the elementary bodies at the early stage of infection purely speculative. It should be noted that these latter irregularly shaped bodies, which have been considered here to be elementary bodies, differ in appearance from structures that have been interpreted as mitochondria in epidermal cells and further that they have never been seen in thin sections from control material from the epidermis examined with the electron microscope.

Summary. Thin sections of *Molluscum contagiosum* lesions from man have been examined with the electron microscope. Cells were seen in the prickle cell layer of the epidermis which contained only a very few cytoplasmic elementary bodies. These are considered to be early infected cells. Cells were seen with varying numbers of elementary bodies scattered through the cytoplasm while others were entirely filled with elementary bodies forming a mature inclusion. The elementary bodies in the early infected cells differed somewhat in shape from most of the elementary bodies seen in the larger inclusions. The mature inclusion bodies were divided into locules by septa. The locules contained mature elementary bodies. The elementary bodies appear to form from the material composing the matrix of the septa by a process of segmentation and condensation. The elementary bodies themselves were sectioned and appeared in some instances to have a formed cortex with a less dense interior.

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Received July 24, 1951. P.S.E.B.M., 1951, v77.

Chemical Differentiation of Cartilage and Bone. II. Sulfatase Activity.* (18945)

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The presence of the polysaccharide, chondroitin sulfuric acid, has been recognized in cartilage for many years. From time to time the possible role of this material in the calcification mechanism of cartilage has been raised. That calcium might be selectively bound to the organic portion of cartilage was suggested a number of years ago by Freudenberg and György(1). More recently the hypothesis(2-4) has been brought forward

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* Aided by grants from Mead Johnson and Co., and the Damon Runyon Cancer Fund.

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that certain changes, *i.e.* depolymerization of chondroitin sulfuric acid, might take place in the calcification zone; as a result certain groupings would be made available to bind calcium. So too, liberation of sulfate might lead to local changes in pH which might affect the solubility of calcium phosphate. In addition, since there is a pronounced reduction in the sulfate content of cartilage as it is transformed into bone(5), we have felt it worthwhile to investigate costal cartilage, particularly the calcifying area, to determine whether any sulfatase activity could be demonstrated. The method is based on the use of p-nitrophenyl sulfate, a colorless compound, which in the presence of sulfatase is converted into yellow p-nitrophenol(6).

TABLE I. Phenolsulfatase Activity of Cartilage, Bone and Periosteum.

Tissue	No. subjects	Phenol-sulfatase units*
Costal cartilage,† human	10	0
” ” † dog	3	0
Rib shaft & marrow elements, human	10	1.2
Rib shaft & marrow elements, dog	3	.8
Rib shaft, alone, human	10	0
” ” ” dog	3	0
Periosteum, human	10	.2
” dog	3	.05

* Per mg wet wt; see text for definition of unit.
† All stages of differentiation(8).

Material and methods. P-nitrophenyl sulfate was prepared according to the methods of Burkhardt and Lapworth(7) and Huggins and Smith(6). I am indebted to Dr. Norman Weisman for this synthesis. Tissue was obtained at autopsy from infants and young children, dying of a variety of causes, and from puppies. Slices of costal cartilage were made beginning in the most undifferentiated region and continuing to the area of calcification by the method previously described(8). Slices of bone with and without marrow elements, the latter being removed by a high pressure jet of water, and strips of periosteum were also assayed. The substrate consisted of p-nitrophenyl sulfate, .005 M, 1 cc and .5 N acetate buffer, pH 5.8, 2 cc. The moist tissue slices or strips were weighed on a torsion balance and placed in the substrate to which a small crystal of thymol was added. The tubes were incubated for 24 hours at 37°C. At this time 2 cc 1 N NaOH were added. Controls were carried through the procedure as described by Huggins and Smith (6). The intensity of color was measured at 420 mμ in a Coleman Junior Spectrophotometer using a standard calibration curve prepared from recrystallized p-nitrophenol. The results are expressed in units, 1 phenolsulfatase unit being defined as the amount of enzyme which produces the color equivalent to 10 γ of p-nitrophenol in a volume of 5 cc in 24 hours at 37°C in .5 N acetate buffer, pH 5.8, the substrate concentration being .005 M. The activity of the system was demonstrated by takadiastase which, of course, exhibits sulfatase activity(6).

Results. In Table I are shown the results of determinations performed on the various tissues already mentioned. No activity is found in cartilage until the zone of hypertrophic cells being invaded by capillaries is reached. However, when the blood elements in this area are removed activity disappears. So too, bone devoid of marrow elements exhibits no activity. Periosteum is more difficult to free of blood; this doubtless explains the presence of slight activity in washed specimens.

Discussion. It is apparent from the foregoing that there is no demonstrable sulfatase activity in cartilage and in bone freed of marrow elements for the substrate which has been employed. From studies reported in the literature which have been carried out with other tissues and substrates it would appear reasonable to consider "sulfatase" a non-specific enzyme which is able to hydrolyze sulfate from a variety of organic compounds,

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one of which is chondroitin sulfuric acid(9). The absence of activity in cartilage is of further interest because of the recent demonstration of the uptake of S^{35} by epiphyseal cartilage(10) and its incorporation into chondroitin sulfate(11).

Summary. Using p-nitrophenyl sulfate as substrate no sulfatase activity could be demonstrated in costal cartilage at varying stages of differentiation or in bone providing no red blood cells or marrow elements were present.

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Received July 25, 1951. P.S.E.B.M., 1951, v77.

Preferential Incorporation of Formate Carbon into Leukemic Blood Cells as Indicated by Autoradiography.* (18946)

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Demonstration of the incorporation of C^{14} into individual blood cells was first accomplished by Boyd, *et al.*(1) who showed by means of autoradiograms that the alpha carbon atom of glycine is taken up most rapidly by rat lymphocytes. The polymorphonuclear leukocytes incorporated C^{14} from glycine less rapidly than the lymphocytes and the circulating erythrocytes were considerably less avid in the utilization of the glycine carbon. The most obvious explanation for this difference in C^{14} content of the different cell types at 25 hours after injection appears to be the difference in their rate of formation (synthesis employing the alpha carbon of glycine as a precursor) and disappearance from the peripheral blood.

In the light of these results, it appeared of interest to study hematopoiesis in leukemic animals using the autoradiographic technic. If we assume that precursors of the various types of blood cells and the mature blood cells are exposed to the same order of concentration of a labeled compound, preferential incorporation by neoplastic cells suggests (1) a difference in the rate of synthesis (if the labeled compound or a degradation product is a metabolite) or (2) qualitative metabolic differences between the formed normal or neoplastic cells.

As a first approach to the study of hematopoiesis in experimental leukemia utilizing the autoradiographic technic, C^{14}-labeled sodium formate has been employed. Formate has been reported to be a precursor of the 2- and 8-carbon atoms of desoxyribose nucleic acid (DNA) and ribose nucleic acid (RNA) guanine and adenine and the 5-methyl carbon of DNA thymine(2). Also, formate carbon has been shown to appear in the alpha carbon of serine and in glycogen(3), in the methyl group of methionine, and the methyl groups of choline(4).

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* This work was supported by grants from the National Cancer Institute and the Biology and Medicine Division of the Atomic Energy Commission. We are indebted to Dr. G. Andrews of the Oak Ridge Institute of Nuclear Studies and Dr. J. H. Burchenal, Sloan-Kettering Institute for Cancer Research, for examination of certain blood smear autoradiograms.

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Experimental. Transplanted Ak-4 leukemia in Akm strain mice has been used in the present experiments. In this leukemia the number of abnormal forms in the differential counts parallels the rise in the total leukocyte count(5,6). "The immature cells characteristic of Ak-4 leukemia were unlike the lymphocytes of normal mice. The young forms most closely resembling prolymphocytes were the largest cells observed and were often two or three times the size of neutrophils. The shape of the prolymphocytes varied from round to oval." With Wright's stain, the cytoplasm ranged from grey-blue to a darker intense blue and was usually coarse, uneven, and vacuolated. The appearance of the chromatin varied, sometimes slightly clumped, more typically very clumped and pyknotic(5). Burchenal(5) further observed in the advance stages of this disease an occasional elevation of mature polymorphonuclear cells. However, because of the staining reactions, general morphology, and complete lack of intermediate myeloid forms, the term "prolymphocyte" was believed most applicable to these leukemic cells.

In our first experiments, 4 leukemic mice of about 2 to 3 months of age were injected intraperitoneally with 4.0 μc each of $HC^{14}OONa$. These mice were in the sixth day of the disease (transplanted Ak-4 leukemia) and had total leukocyte counts of the order of 100,000. Blood was taken directly from the heart of injected mice at 6 hours and the packed cells were washed twice with inactive heparinized mouse plasma. This washing was necessary to remove active sera which would cause confusing background in the final autoradiogram. The cells were then diluted with inactive mouse plasma (5:1) prior to making smears in a darkroom. Smears were made directly on Eastman NTB plates, emulsion thickness 10 μ, air dried, and exposed for 6 weeks(1). After exposure, the cells were fixed with methyl alcohol and the NTB plates were developed 2 or 20 minutes (the latter to bring out beta tracks) in full strength D-19 at 20°C. The plates were then cleared in 10% sodium thiosulfate and the cells stained with Wright's stain.

In this first study, the autoradiograms which resulted from injection of 4.0 μc per mouse were faint even after 6 weeks' exposure. These autoradiograms were examined by an arbitrary technic which entailed counting silver grains immediately surrounding the individual cells and comparing with the background. An eyepiece reticle which divided a given field at 970X into 10 μ squares was placed in the microscope. Then, centering a given cell at the intersection of a 4-square area, the silver grains in the 4 squares (20 μ x 20 μ) were counted while focusing up and down in order to observe grains in different planes. The background was counted in 26 different fields where there were no cells and the average number of grains per mm^2 determined. This average was subtracted from the values obtained around the cells examined giving a net count in the area immediately around the respective cells. The difference between background and prolymphocyte grain counts was statistically significant, (Table I). These counts showed the prolymphocytes to have on an average about 7 times as many silver grains (net particles per mm^2) in the area immediately around the cell as did the small lymphocytes. The small lymphocytes and polymorphonuclears had grain counts slightly but not significantly above background.

In a second series of experiments, mice with advanced Ak-4 leukemia were injected I.P. with 100 microcuries each of C^{14}-formate and blood smears made at one hour. After varying periods of exposure, the NTB plates were developed, stained, and examined. With this level of carbon 14, the autoradiograms were so pronounced at one week after development that it was relatively simple to classify cells as grossly positive or negative with regards to their radioactivity. In NTB plates exposed for a period greater than 2 weeks, there were so many silver grains associated with the cells that they interfered with the morphologic classification. In view of this, it was decided to use cell diameter in addition to morphology in classification. The polymor-

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TABLE I. Grain Counts Immediately Around Blood Cells from a Mouse Injected with $HC^{14}OONa$ (4 μc).

	No. of fields counted	Range of counts	Avg count per mm²	σ	Avg net count per mm²*
Background	26	2,500–27,400	13,400	5,000	
Prolymphs	12	24,900–90,000	48,000	15,500	+35,400
Lymphs	17	2,700–20,600	18,800	7,000	+5,400
Polys	13	5,400–31,400	19,000	8,300	+5,600
Erythrocytes	12	2,700–18,400	11,600	4,000	—1,800

* Average net count per mm² = average counts—background.

TABLE II. Per Cent Positive Blood Cell Autoradiograms Observed in Mice with Advanced Ak-4 Leukemia at One Hour after Injection of C14-Formate (100 μc).

				Differential count				% positive autoradiograms			
Mouse No.	Observer	Cell origin	Total WBC	Polys	Prolymphs	"Large" lymphs	Lymphs	Polys	Prolymphs	"Large" lymphs	Lymphs
2	1	Blood	45,000	20	18	11	51	0	89	36	2
	2			14	13	9	53*	0	100	50	5*
	3			18	20	10	52	0	94	50	1
	4			19	21	10	52	0	90	35	2
	5			—	—	—	—	0	82	78	6
3	1 and 4	Blood	64,000	16.5	19	14	78	0	82	78	6

* This value included 10 "unidentified" negative cells in the range of 6-9 μ diameter.

Note: All observations are on random fields and include counts of 100 cells or more. The classification " 'large' lymphs" includes lymphoid cells of 10-12 μ (difficult to classify morphologically because of the presence of silver grains in the nuclear track emulsion). A large percentage of these cells are believed to be leukemic.

phonuclear cells were easily classified since they gave uniformly negative autoradiograms. Non-radioactive leukemic blood smears on NTB plates carried through the development and staining procedure showed that normal lymphocytes ranged from about 6 to 9 μ in diameter. Prolymphocytes (leukemic cells) ranged in size from about 10-18 μ in diameter. There were a small percentage of cells in the lymphocytic series which were intermediate in size (10-12 μ) and which could not be unequivocally classified as normal large lymphocytes or leukemic cells. It was therefore considered most practical for this initial work to report these autoradiograms on the following basis:

Type		Criterion
Polymorphonuclears	-	Identifiable by morphology
Lymphocytes	-	6-9 μ diameter
'Large' lymphocytes	-	10-12 μ "
Prolymphocytes	-	12-16 μ "

It is believed that on the basis of the morphology of these "large" lymphocytes, a goodly percentage may be leukemic cells. In any event, under this classification, little opportunity prevails for mistaking the prolymphocytes (leukemic cells) and the normal lymphocytes.

Five observers have now studied a large number of autoradiograms of leukemic mouse blood taken at one hour after I.P. injection of 100 μc of $HC^{14}OONa$. The agreement on the per cent of active cells of various types has been good. The results of these observations are given in Table II. Seven additional smears on NTB emulsion made from blood taken from mouse No. 2 were examined by two different observers as were duplicate smears from four additional leukemic mice. The additional results obtained were in good agreement with those reported in Table II. These autoradiograms were progressively darker with increased exposure and on 20-minute development in full strength D-19, beta tracks were in evidence. Typical autoradiograms are shown in Fig. 1 and 2.

The possibility of chemical fogging in these

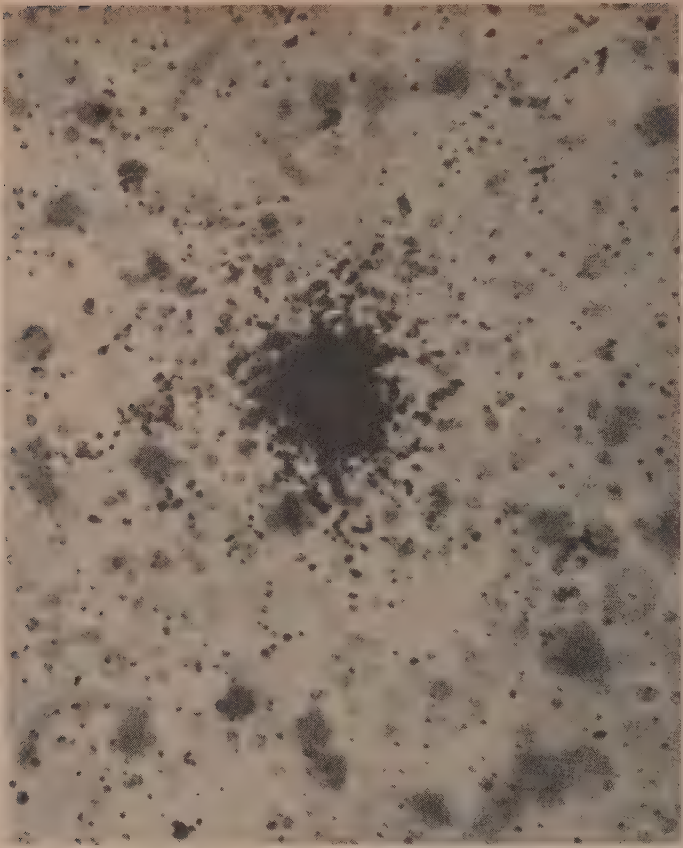

FIG. 1. A positive autoradiogram of a leukemic prolymphocyte showing the peripheral concentration of silver grains obliterating the outline of the cell. Blood taken from a leukemic mouse 1 hr after injection of 100 μc of C^{14}-formate.

studies has received considerable attention. In no instance have we observed chemical fogging in control smears, from normal or leukemic mice, made in the same manner and exposed and developed under the same conditions. To date 65 control smears have been made on blood from 18 leukemic mice and 49 control smears have been made on blood from 11 normal mice.

Discussion. From the results obtained, it appears that at one hour after injection of C^{14}-formate into mice with Ak-4 leukemia most (approximately 90%) of the prolymphocytes (leukemic cells) contain considerable amounts of the active atom, while the more mature cells are relatively inactive. A small percentage of the cells classed as normal lymphocytes were active though generally much less so than the prolymphocytes. In no instance in these one-hour experiments have we seen positive polymorphonuclear leukocytes, erythrocytes, or thrombocytes. These observations could be explained on the basis of the very rapid mitotic division and accompanying synthetic activity which must be existent in the leukemic hematopoietic tissue. Such an assumption would require the accompanying conclusion that there has been an almost complete turnover of leukemic cells within the period of these experiments while very few normal lymphocytes, polymorphs, or erythrocytes have been produced. A second and perhaps more reasonable conclusion which might be drawn from these data is the exist-

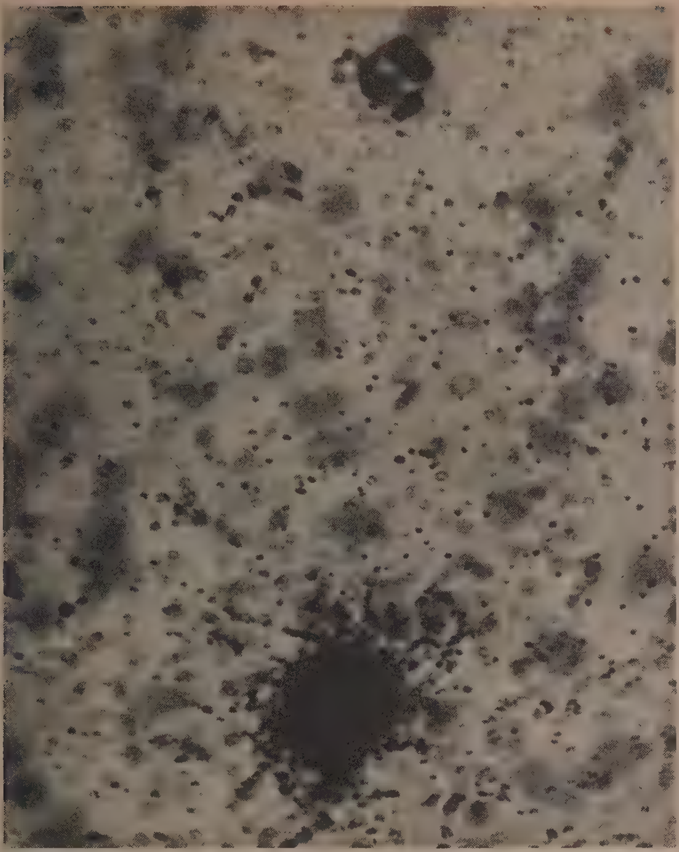

FIG. 2. A positive autoradiogram of a leukemic prolymphocyte and a negative polymorphonuclear leukocyte. Negative erythrocytes appear as shadows in the background. Blood from a leukemic mouse 1 hr after injection of 100 μc of C^{14}-formate.

ence of a profoundly different rate of formate metabolism in formed leukemic and normal cells.

Any attempt to explain the present data on a biochemical level must take into consideration the level and activity of citrovorum factor coenzyme (thought to be involved in formate transfer) in normal and leukemic cells. Bethell (7) has observed that leukemic cells are considerably higher in citrovorum factor than are normal white blood cells. In view of the fact that: (a) formate is a precursor of nucleic acid moieties(2) and carbon atoms of certain amino acids(3) and other important biochemicals(3,4), (b) folic acid antagonists inhibit the conversion of folic acid to citrovorum factor (8); cause the build-up of 4-amino-5-carboxamido-imidazole in bacteria(9); and inhibit incorporation of formate into nucleic acids(10), (c) folic acid deficiencies inhibit incorporation of formate into proteins(11), (d) folic acid inhibitors preferentially affect

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production of leukemic cells(5,6) and increase the life span of leukemic mice(12,13), (e) the anti-leukemic action of folic acid antagonists may be reversed by folic acid or citrovorum factor(14,15), and partially reversed by DNA or B_{12}(16,17), certain possibilities pertinent to the present discussion would seem to exist. Certainly the present data indicate that leukemic cells are geared to a more active formate metabolism than are normal white blood cells. Since inhibition of formate metabolism via the citrovorum factor coenzyme preferentially inhibits proliferation of leukemic cells, it might be suggested that these malignant cells are utilizing their supplies of this coenzyme to capacity while normal blood elements possess a marginal reserve of this formylating system. If this were true, folic acid antagonists might provide specific anti-leukemic action *in vivo* even though the antagonist concentration in the malignant and normal cell was of the same order because of differential demands on the formylating system.

Further studies are under way in an attempt to determine the significance of this difference in formate incorporation by normal and leukemic cells.

Summary. At one hour after injection of 100 μc of C^{14}-formate into mice with advanced Ak-4 leukemia, it has been shown by means of blood smear autoradiograms that about 90% of the leukemic cells are highly active as compared to about 5% of the more mature lymphoid elements. No active polymorphonuclears, erythrocytes, or thrombocytes were observed within one hour.

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Received July 27, 1951. P.S.E.B.M., 1951, v77.

Measurement of Total Body Chloride.* (18947)

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The chloride ion has been considered to be confined for the most part to the extracellular fluid. It has been accepted, nevertheless, that the chloride ion penetrates certain cell walls, in particular the wall of the erythrocyte, the gastric mucosa, the small intestine, the colon mucosa, the liver cell to be excreted in the bile, the cells of sweat and salivary glands, and of the kidney tubule. Precisely how much of the chloride is included in the intracellular water has not been appreciated until it was shown recently that inulin was distributed through a volume of fluid which more closely approximated the anatomical extracellular volume than did the larger "chloride space"(1). When the volume of fluid in which inulin is distributed is taken to be the measure of extracellular fluid, the difference then between the chloride contained in this body of fluid and the total chloride in the body represents intracellular chloride.

Bromide is distributed in tissues in much the same manner as chloride(2-4) and has,

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* This investigation was supported in part by research grants from the National Institutes of Health, Public Health Service, and the Public Health Research Institute of the City of New York.

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therefore, been used as a measure of what has been called "chloride space"(5). Since it is now apparent that a large proportion of chloride is not extracellular, it is better to consider the bromide as being diluted in the total chloride of the body rather than in a certain volume of water. The dilution of bromide is then a measure of total body chloride in the same way that the dilution of an isotope is a measure of the total quantity of the isotopically normal element; for example, the dilution of radioactive potassium to measure total body potassium(6)[†].

Methods. Bromide was administered to hospital patients and personnel in amounts calculated to achieve plasma levels of 2 mEq per liter. To subjects without edema bromide was administered intravenously from a burette as a 4% sodium bromide solution. Subjects with edema received the bromide orally as potassium bromide in capsules. In normal individuals, plasma was sampled after five hours and in those with edema, after 24 hours. Evidence that equilibrium has been established at these times for normal and edematous individuals has been previously documented by Brodie, Brand, and Leshin(5). In 5 edematous patients of the present series, concentration of bromide in ascitic fluid and plasma was in good agreement 24 hours after administration. The urinary excretion of bromide which is small (maximum of 3% of the dose in edematous patients) was measured during the 24 hours equilibration and the amount administered was corrected for that excreted. Bromide was determined by the method of Brodie and Friedman(10); the plasma values were corrected for plasma blanks measured in specimens drawn prior to the administration of bromide. Chloride was determined by the method of Wilson and Ball(11). The bromide administered was considered part of the total body chloride and the total plasma halide determined by precipitation with silver nitrate was not corrected for the small amount of bromide present. Total body chloride was calculated from the relationship

$$\frac{\text{Concentration of chloride in plasma}}{\text{Concentration of bromide in plasma}} = \frac{\text{Total body chloride}^{\ddagger\S}}{\text{Retained bromide}}.$$

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[†] There are some minor qualifications. One, only part of the chloride in the central nervous system is included because of the barrier to the passage of bromide into the spinal fluid(7). On the other hand the ratio of bromide to chloride in red cell water is somewhat higher than that in plasma water (8, 9). These two discrepancies tend to nullify each other.

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[‡] Total chloride cannot be calculated from urinary concentrations of chloride and bromide after equilibration as might be done with an isotopic element. The kidney tubule preferentially reabsorbs bromide to chloride. The U/P ratio of chloride is 1.5 times the U/P ratio of bromide (mean of 20 comparisons none below 1.0).

[§] Thiocyanate presumably distributes in tissues in proportion to their chloride content although direct comparisons of chloride with thiocyanate in tissues have not been reported in the literature(12). Twenty-two comparisons were made between the total chloride as measured by thiocyanate and the total chloride as measured by bromide. The comparisons were made in a group consisting of 16 normal individuals and 6 edematous patients. The ratio of thiocyanate measurement to bromide measurement was 0.99 (mean of 22 comparisons). The individual thiocyanate measurements ranged from —24 to +18% of the bromide; on the average the thiocyanate differed from the bromide by ± 9.5%. Although thiocyanate is much easier to analyze than bromide, bromide was considered preferable for measurement of total body chloride because of the reported metabolism and protein binding of thiocyanate(13, 14, 15).

TABLE I. Total Body Chloride in Males and Females.

Wt group, kg	No. of subjects	Mean age, yr	Mean wt, kg	Mean plasma chloride, mEq/l	Mean total chloride, mEq	Mean chloride/kg body wt, mEq/kg
Males						
40–49	3	58	46.6	105	1530	32.9
50–59	6	52	56.8	103.7	1997	35.2
60–69	9	39	64.9	103.5	1974	30.6
70–79	5	42	76.8	104.9	2354	30
80+	3	36	82.3	103.5	2305	27.9
Mean	(26)		65.2		2039	31.6
σ					318	3.8
Females						
40–49	4	42	47.2	104.6	1558	32.9
50–59	6	27	54.8	104.9	1600	29.3
60–69	6	34	63.2	104.8	1812	28.7
70–79	5	42	75.5	104.4	2026	26.9
80+	5	33	97.2	108.6	2484	25.8
Mean	(26)		67.7		1894	28.6
σ					355	3.3
Combined group males and females						
Mean	(52)		66.6		1967	30.1
σ					345	3.9

Observations. Total body chloride in normal individuals. The normal individuals (26 males, 26 females) consisted of hospital personnel and patients without evident abnormality in their degree of hydration. The mean total body chloride of the males (average weight 65.2 kg) was 2039 mEq, that of females (average weight 67.7 kg) was 1897 mEq (Table I). Using the volume of distribution of inulin as the best measure of extracellular fluid volume, an estimate may be made as to what portion of the total body chloride is inside and what portion is outside the cell. The average extracellular fluid volume, as measured by dilution of inulin is approximately 15% of body weight(1,16-18) or for this group (average weight 66 kg), 10 liters and contains 110 mEq of chloride per liter.¶ Extracellular fluid thus accounts for only 1100 mEq of the total chloride found in the body. The difference between the extracellular chloride and the total body chloride is about 900 mEq, which represents chloride within the cells. It is evident that almost half of the chloride in the body is intracellular. Of interest is the relationship of total body chloride to body weight. In both male and female subjects, the chloride content per kg of body weight tended to decrease as the weight increased. Presumably fat which contains no chloride, or muscle in which the concentration of chloride is quite low compared to other tissues, forms a larger proportion of the body as body weight increases. The average chloride in the males was 32.6 mEq and in females 28.6 mEq per kg. The smaller amount of chloride per unit weight in the female is to be expected since the female contains proportionately more fat(19).

Total body chloride in patients with edema. Twenty patients with abnormal fluid depots were grouped according to the extent of their edema which was rated 1 to 3+ (Table II). The average amount of chloride per kg in each group increased with the extent of the edema. In the group with 1+ edema the

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TABLE II. Total Chloride in Patients with Clinical Edema.

Degree of edema	Subject	Diagnosis*	Sex	Wt, kg	Plasma chloride, mEq/l	Total chloride, mEq	Chloride per unit wt, mEq/kg
1+	A	CHF	F	50	101.8	1950	39
1+	B	"	M	51	104.5	2010	39.4
1+	C	"	M	58.9	108.2	2860	48.6
1+	D	"	M	75.7	94.5	2770	36.6
		Mean		58.9	102.2	2398	40.9
2+	E	"	F	45.8	103.1	2260	49.3
2+	F	NEP	M	49.1	101	2180	44.4
2+	G	CHF	F	51.2	113.5	2980	58.2
2+	H	NEP	M	55.6	102	2710	48.7
2+	I	CHF	F	56.8	103.9	2200	38.7
2+	J	"	F	63	106	2260	35.8
2+	K	"	F	76	106	2580	33.9
		Mean		56.8	105	2453	44.2
3+	L	"	F	40	85.6	1530	38.3
3+	M	"	F	45	89.7	2120	47.2
3+	N	CIR	M	50.9	107.8	3270	64.4
3+	O	"	M	63	103	2820	44.8
3+	P	CHF	M	67.9	101.8	4740	69.9
3+	Q	CIR	M	68	112	2840	41.9
3+	R	CHF	M	68.3	98.4	3150	46.2
3+	S	NEP	M	73.2	104	2970	40.6
3+	T	CIR	M	78.5	98	3160	40.2
		Mean		61.6	100	2955	48.2

* CHF, congestive heart failure; NEP, nephritis; CIR, cirrhosis.

chloride content averaged 40.9 mEq per kg with 2+ edema 44.2 mEq per kg, and with 3+ edema 48.2 mEq per kg. It may be noted also, that the chloride content of an individual patient may differ markedly from the others in his group. For example, the patient (L) who had been on a low sodium diet (500 mg) and had received frequent injections of mercurial diuretics contained less chloride than the others in the group. High values occurred in patients who had received ammonium chloride (C, G, P). Although the data indicate that the total chloride increased with the extent of the edema, the individual values must be interpreted in terms of the clinical state and prior therapeutic management.

Discussion. Estimates of the chloride content of normal man have been made in the past and the value of 2400 mEq commonly accepted in the literature(20,21) is quoted from work published in 1910 by Magnus-Levy(22). He analyzed the concentration of chloride in various tissues obtained from a 37-year-old male suicide, and multiplied these values by the individual tissue weights proposed by Bischoff in 1863(23). On this basis, he was able to account for 27.4 g (770 mEq) of chloride in the tissues analyzed. He accepted as well known the amount of blood and its chloride content. Blood is recorded as 4.5% of body weight containing 3.5% chloride which brings the total body chloride content to 42.3 g (1190 mEq). For the concentration of chloride in the skin, skeleton, and adipose tissue he "annexed" the values from Nencki (1894) and Wahlgren (1909) who performed their analyses on dog tissues (2,24). The "rest" is 1.32 kg containing 2% chloride. On this basis he estimated total

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body chloride as 85 g (2400 mEq). Considering the evidence from which this value of 2400 was synthesized it is a really good estimate for total body chloride compared with the value of 2000 mEq calculated from the dilution of bromide.

The amount of chloride per unit weight found in man may be compared with that found in other mammals. Weir(25) using the dilution of bromide as a measure of total body chloride in nephrectomized animals, reports 33.3 mEq of chloride per kg as a mean of 9 dogs, 34.4 mEq per kg as a mean of 10 cats, and 30.2 mEq per kg as a mean of 10 rabbits. These values are in agreement with those obtained by the direct analysis of chloride in the total animal. Rosemann extracted the entire animal with alkali and reports 33.6 mEq of chloride per kg as a mean of 3 dogs(26). Harrison, Darrow, and Yannet homogenized the entire animal and report 33.6 mEq per kg as a mean of 2 dogs, 31.2 mEq per kg as a mean of 3 rabbits, and 31.7 mEq per kg in 2 monkeys(27). In the group of 52 individuals in this report the average chloride found was 30.1 mEq per kg. The amount of chloride per unit weight in man, is then quite close to that found in the dog, cat, rabbit, and monkey.[‖]

The present data indicate that about half of the body chloride is within the cells. When a large portion of the chloride is intracellular a change in total chloride does not necessarily represent a change in the volume of extracellular fluid as the change or any portion of it may have taken place within the cells.

If the 900 mEq of intracellular chloride is evenly distributed throughout approximately 25 liters of intracellular water(1), the intracellular concentration of chloride may be estimated at 36 mEq per liter. Of course there is considerable variability of the chloride concentration in the intracellular water of various tissues. In muscle cell water the concentration of chloride seems negligible, whereas in skin it is at least 110 mEq per liter(29). The concentration of chloride in red cell water is 75 mEq per liter(30). In view of this variability it is difficult to give physiological meaning to the "average" value of 36 mEq per liter. It is evident from this data that the volume of dilution of bromide as it is ordinarily calculated does not represent in any sense a volume of water in which chloride is evenly distributed since the anion is present in different tissue cells in varying concentrations.

Summary. Total body chloride was measured in normal man by the dilution of bromide and it has been estimated that nearly half the total body chloride is intracellular.

The authors are indebted to Marvin Fink and Roslyn Jackenthal for their technical assistance.

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[‖] Recently Burch(28) using the dilution of isotopic Cl^{36} reports 42.8 mEq of chloride per kilo as an average of 5 dogs, the range of values for these 5 dogs, however, was rather wide 29.0 to 60.8 mEq/kg.

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Received April 17, 1951. P.S.E.B.M., 1951, v77.

A Heterocyclic-Substituted Sulfone with Activity against *Mycobacterium tuberculosis.* (18948)

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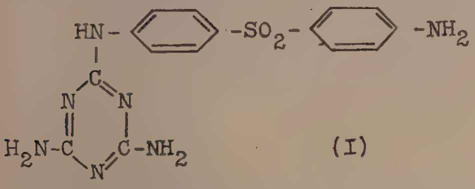
N-substituted derivatives of 4,4′-diaminodiphenyl sulfone are thought to owe their *in vivo* antibacterial activity to the catabolic removal of the substituent groups, since the N-substituted compounds generally have low *in vitro* activities and lead to the presence of diazotizable amine in the blood after administration to animals. High potency is believed to be encountered only when both amino groups of the parent sulfone can be readily liberated. It has been speculated that in the case of N-heterocyclic derivatives catabolic cleavage cannot occur and that such compounds are inactive *per se*(1).

An N-heterocyclic-substituted sulfone (I)



(I)

has now been synthesized, which in contrast to previously reported compounds of its type, is highly active *in vitro* against *Mycobacterium tuberculosis* and *Escherichia coli.*

Synthesis. 4,4′-Diaminodiphenyl sulfone (24.8 g) and an equimolar amount of 2-chloro-4,6-diamino-s-triazine (14.5 g) are permitted to react over a period of 10 minutes in 1 liter boiling 0.35 N hydrochloric acid, according to the method described by Banks and co-workers(2). On cooling to 13°C, a white precipitate of the very sparingly soluble dihydrochloride of bis[p-(2,4-diamino-s-triazinyl-6)-aminophenyl] sulfone is obtained and removed by filtration. The clear filtrate on keeping at 5°C for 72 hours deposits the dihydrochloride of the desired product, p-(2,4-diamino-s-triazinyl-6)-aminophenyl p′-aminophenyl sulfone (I), which is purified by recrystallization from dilute hydrochloric acid. The yield is 13.4 g (31%).

Analysis:
Calculated for $C_{15}H_{15}O_2N_7S$, 2 HCl: N 22.8
Found : N 22.8

This hydrochloride is converted to the corresponding free base with sodium carbonate in 88% yield. The base is a white powder which is only slightly soluble in water, sparingly soluble in dilute hydrochloric acid and soluble in propylene glycol. The product may be diazotized and coupled with phenols. When heated rapidly, the base melts at 240-245°C.

Analysis:
Calculated for $C_{15}H_{15}O_2N_7S$: N 27.4, S 9.0
Found : N 27.3, S 8.8

Microbiological tests. The H37Ra strain of *Mycobacterium tuberculosis* and the B strain of *Escherichia coli* were used. The test media for the two organisms were slight modifications of those described by Dubos and Davis(3) and Wood(4) respectively.

TABLE I. Effect of p-(2,4-diamino-s-triazinyl-6)-aminophenyl p′-Aminophenyl Sulfone (DTS) and 4,4′-Diaminodiphenyl Sulfone (DADS) on Growth* of *Mycobacterium tuberculosis* and *Escherichia coli.*

Molarity of compound in test medium	*Mycobacterium tuberculosis*		*Escherichia coli*	
	DTS	DADS	DTS	DADS
5×10^{-4}	0	0	0	0
1.2×10^{-4}	0	1	0	0
3.1×10^{-5}	2	2	1	0
7.8×10^{-6}	3	3	2	2
0 (controls)	4		3	

* Amount of growth: 0, none; 1, slight; 2, moderate; 3, heavy; 4, very heavy. Incubation period: 2 weeks for *M. tuberculosis,* 2 days for *E. coli,* both at 37°C.

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As shown in Table I, the *in vitro* activity of p-(2,4-diamino-s-triazinyl-6)-aminophenyl p'-aminophenyl sulfone is of the same order as that of 4,4'-diaminodiphenyl sulfone. Since p-aminobenzoic acid (5×10^{-5} molar) was found to reverse completely the antibacterial action of the heterocyclic sulfone (5×10^{-4} molar), the latter presumably acts through a mechanism similar to that reported for 4,4'-diaminodiphenyl sulfone(4).

Summary. p-(2,4-Diamino-s-triazinyl-6)-aminophenyl p'-aminophenyl sulfone has been synthesized and shown to inhibit the growth of *M. tuberculosis* and *E. coli in vitro* at a concentration of 1.2×10^{-4} moles per liter, the inhibition being reversed by p-aminobenzoic acid.

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Received July 24, 1951. P.S.E.B.M., 1951, v77.

Production of Acrodynia in Mice Fed Diets Low in Pyridoxine.* (18949)

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During the course of an investigation on the influence of vitamins of the B complex on the induction of tumors in mice, a fortuitous observation of an acrodynia was made. This condition was cured by the injection of pyridoxine(1). However the diet fed to these mice was low in several of the B vitamins.

The present report describes further studies on the production of acrodynia in mice. The results are of interest because it has been reported that the pyridoxine deficient mouse, in contrast to the rat, does not develop acrodynia(2-5) except with the aid of desoxypyridoxine(6).

Methods and results. Since the original observation of acrodynia was made on female albino mice that were 2-3 months of age at the start of the experiment(1), similar mice were used in the present study. Weanling male mice, capable of rapid growth on a complete diet, were also used. These mice were obtained from a commercial source,[†] and upon arrival were immediately placed in screen bottom cages and were given water and the experimental diets *ad libitum*.

The following 4 diets were used: *Diet A*, a semi-purified diet plus pyridoxine: Glucose monohydrate (Cerelose), 78; ordinary commercial casein, 15; corn oil (Mazola), 2; liver concentrate (Wilson Laboratories 1-20 liver powder), 1; salt mixture (modified Phillips-Hart), 4. This was supplemented with the following vitamins per kg of diet: pyridoxine-HCl, 30 mg; thiamine-HCl, 1.55 mg; biotin, 0.1 mg; inositol, 20 mg; para aminobenzoic acid, 20 mg; choline-Cl, 20 mg; and 6 drops of halibut liver oil premixed with the corn oil. *Diet B*, a semi-purified diet without added pyridoxine: This diet was the same as diet A except that no pyridoxine was added. *Diet C*, a purified diet with low pyridoxine: This diet differed from diet A as follows. The crude casein was replaced by purified casein (Smaco) and the liver concentrate was replaced by an equivalent weight of glucose. The following vitamins were added per kg: pyridoxine-HCl,

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* This investigation was supported by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

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[†] Arthur Sutter, Springfield, Mo. The mice were described as "Rockland Strain."

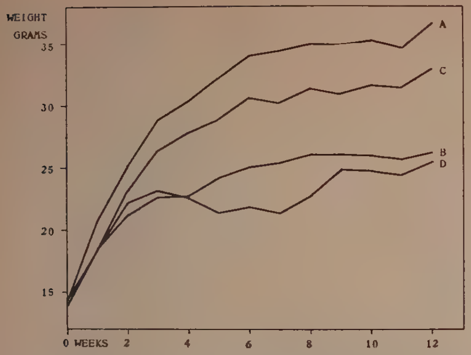

FIG. 1. Growth curves showing the effects of various diets on the growth of weanling male mice.[‡] A. Growth curve of mice fed the non-purified diet plus pyridoxine. B. Growth curve of mice fed the non-purified diet without added pyridoxine. C. Growth curve of mice fed the purified diet with low pyridoxine. D. Growth curve of mice fed the purified diet without added pyridoxine.

0.166 mg; thiamine-HCl, 3 mg; riboflavin, 3 mg; niacinamide, 5 mg; calcium pantothenate, 20 mg; folic acid, 1 mg; para aminobenzoic acid, 250 mg; inositol, 250 mg; choline-Cl, 500 mg; biotin, 0.1 mg; and 6 drops of halibut liver oil. *Diet D,* a purified diet without pyridoxine: This diet was the same as diet C except that no pyridoxine was added. Each of the 4 diets was fed to a group of 8 weanling male mice and to a group of 8 females about 2 months of age, making 8 groups in all.

In Fig. 1 are shown the growth curves of the 4 groups of weanling male mice. Excellent growth was shown by the mice fed the semipurified diet plus pyridoxine (diet A), and the purified diet containing a presumed suboptimum level of pyridoxine (Diet C) allowed good growth. However growth was inferior in mice fed either diet without added pyridoxine (Diets B and D). Since the young adult female mice were nearly full-grown and therefore showed less growth, their weight curves are not shown. The initial weights averaged 23 g and 12 weeks later the average of each of the 4 groups ranged from 26 to 33 g, and the curves of the averages followed the same pattern as was the case for the young growing male mice.

Both groups of mice fed diet A were in good health throughout the experiment and no definite signs of deficiency were noted in the two groups fed the purified diet with a trace amount of pyridoxine, diet C. However, acrodynia was observed between the 8th and 9th weeks in each age-sex group fed diets B and D. The acrodynia affected a greater proportion of the young adult female mice fed the purified diet without pyridoxine (5 of 8 mice) than in the other 3 groups (2 of 8 in each). Furthermore 3 of the 8 weanling mice fed the purified diet without added pyridoxine died between the 7th and 12th weeks.

At the 12th week, pyridoxine therapy was begun on 3 female mice with severe acrodynia that had been fed the purified diet without added pyridoxine. The appearance of these mice became normal within one week by the addition of 30 mg/kg of pyridoxine-HCl to the diet.

Discussion. The acrodynia displayed by the mice in these experiments resembled that described by Birch, Gyorgy, and Harris(7) in the rat. The ears of these mice were red and swollen, and the paws were scaly and even raw. There was dermatitis of the nose which often extended beyond the eyes. The tail was not affected, although scaliness sometimes appeared at the base. An ataxic gait involving the hind legs was also observed. The mice appeared anemic but the hemoglobin was not determined.

This syndrome was related specifically to a low dietary level of pyridoxine. As little as 0.166 mg of added crystalline pyridoxine-HCl per kg of the purified diet protected the mice from acrodynia and resulted in a good growth response. In contrast, mice fed the semipurified diet without added pyridoxine developed acrodynia, yet this diet has been calculated to contain over three times as much pyridoxine-HCl as the purified diet, namely 0.51 mg/kg. Perhaps the pyridoxine of the liver powder and casein as determined microbiologically is not all available to the mouse.

[‡] The increase in the average weight of the surviving mice of group D between the 7th and 8th, the 8th and 9th, and the 11th and 12th weeks is accounted for by the death of one mouse during each of these weeks.

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No explanation is offered for the appearance of acrodynia in our experiments in contrast to the usual freedom in mice from acrodynia during pyridoxine deprivation. By inspection of the conditions of previous studies of B_6 deficient mice together with the variables introduced in our experiments, no definite correlation could be established relating the composition of the diet, age or sex of the mice, the probable pyridoxine content of the diet, levels of other vitamins, etc., to the protection from, or appearance of, acrodynia. The syndrome has appeared in the mice without fail on 3 different occasions that were so spaced in time (February, May, and September) that such factors as season and humidity were probably not involved. Only one strain of mice was tested, and it may be that strain differences play an important role in the appearance of acrodynia.

Summary. 1. Pyridoxine deficiency was produced in weanling male and in young adult female mice fed (a) a purified diet containing 9 other crystalline B vitamins, and (b) a semipurified diet containing a low level of liver powder. 2. Acrodynia appeared among both the weanling and young adult mice fed these 2 diets. 3. As little as 0.166 mg of added crystalline pyridoxine-HCl per kg of the purified diet protected the mice from acrodynia and resulted in a good growth response by the weanling mice.

Received July 16, 1951. P.S.E.B.M., 1951, v77.

Role of Bile and Pancreatic Juice in Production of Esophageal Erosions and Anemia.* (18950)

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Numerous studies have been carried out on the ability by trypsin to digest living tissues and on the toxic effect of bile salts on certain tissues. Claude Bernard(1) showed that the leg of a living frog was digested away when introduced into the stomach of a dog. Rosenbach(2) produced hemorrhages on the tongue of a frog by the local application of a solution of active trypsin. Langenskjöld(3) found he could cause ulceration and necrosis of urinary bladder mucosa in the dog by perfusing it with pancreatic juice, but not to the degree produced by gastric juice. Kirchheim and Böttner(4) found no changes in the mucous membrane of the urinary bladder, esophagus or intestine after exposure to active trypsin solution for 10 hours. Necheles and associates(5) and Dragstedt and associates(6) implanted various organs such as pancreas, kidney, liver and omentum into the duodenum exposed to active pancreatic ferments and found in most cases no digestion of these organs. Rywosch(7) was one of the first investigators to point out the toxic effect of bile salts on various tissues. Opie(8) and Flexner(9) showed the destructive effect of bile

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* This study was supported by the following grants: 1. The United States Public Health Service (Ulcer Project), Contract RG1026-C4; 2. The Augustus L. Searle Fund; 3. The Austen S. Cargill Fund; and 4. The Graduate School of the University of Minnesota.

† U.S.P.H.S. Fellow.

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and bile salts on the pancreas, and Smith(10) on the gastric mucosa. The present study was prompted by recent work demonstrating the susceptibility of the esophagus to injury by acid-peptic juice(11), and by the circumstance that patients who undergo total gastrectomy often complain of symptoms occasioned by the regurgitation of bile into the esophagus.

Present study. Two sets of experiments were carried out, the first on cats, and the second on dogs. In cats weighing 1.4 to 3.5 kg under nembutal anesthesia, the esophagus was perfused with mixtures of bile and pancreatic juice, bile alone, pancreatic juice alone and intestinal juice collected from dogs. Also, perfusing solutions of human bile and of bile salts in a concentration of 0.65 g of bile salt for each 5 cc of normal saline were used. A cannula was tied into the cervical esophagus and another into the stomach just beyond the esophago-gastric junction. The perfusing solution at a temperature of 38° was dripped from a burette into the proximal cannula at a rate of 25 cc per hour until the cat died, but no longer than 8 hours. The pressure of the solution in the esophagus was not allowed to exceed 20 cm of water. The juice used for the perfusions was collected from duodenal fistulae in dogs, some of which drained bile and pancreatic juice, and others of which drained only pancreatic juice. Bile was collected from dogs by means of a cholecystostomy tube, and succus entericus was obtained from a 45 cm loop of duodeno-jejunum. Human bile was collected from patients at the time of autopsy, or from patients who had been on T-tube drainage of the common bile duct for not more than 2 days. The mixtures containing pancreatic juice were collected at frequent intervals and frozen to prevent loss of tryptic activity. The activity of these juices was tested at the time of each perfusion by the modified method of Fermi(12). None of the bile, collected either from dogs or humans, showed tryptic activity.

The second set of experiments was carried out on mongrel dogs weighing 9 to 18 kg. Duodeno-esophagostomies, cholecystojejuno-esophagostomies, jejuno-esophagostomies, and total gastrectomies with end to end duodeno-esophagostomies were made to cause diversion or regurgitation of bile and pancreatic juice, pancreatic juice alone, bile alone, and succus entericus into the esophagus. The dogs were esophagoscoped within 8 to 14 days after the initial operation and were followed at frequent intervals by esophagoscopy to ascertain the degree to esophagitis developing. Complete blood counts were taken each week, and stools were studied for occult blood during periods when the dogs were on a meat free diet. The dogs were sacrificed after a period of 1 to 3 months. To rule out postmortem autolytic changes produced by the bile and pancreatic juice, only those dogs autopsied immediately after death were counted in the series.

Results. A. Cats. The criteria for grading the degree of esophagitis produced in cats are outlined in the footnote of Table I. From this table it can be seen that, in general, perfusions with mixed bile and pancreatic juice caused the most severe changes, and pancreatic juice alone caused only minimal changes.

TABLE I. Perfusion of Esophagus in Live Cats with Various Juices.*

Type of juice	No. of cats	Severe changes	Minimal changes	No changes
Mixed bile and pancreatic juice	19	12	6	1
Bile only	14	7	4	3
Pancreatic juice only	10	1	9	0
Sodium taurocholate or sodium glycocholate	8	8	0	0
Jejunal juice	5	0	0	5
Human bile	6	5	1	0

* Criteria for grading esophagitis in cats: *Severe esophagitis*: 1. definite ulcerations; 2. marked discoloration of mucous membrane with many erosions and petechiae; 3. fluid in mediastinum or thoracic cavity. *Minimal esophagitis*: 1. a few erosions and petechial hemorrhages; 2. discoloration of mucous membrane in addition to No. 1; 3. no ulcers, no fluid in mediastinum or thoracic cavity. *No changes*: 1. mucosa merely bile-stained. None of changes outlined above.

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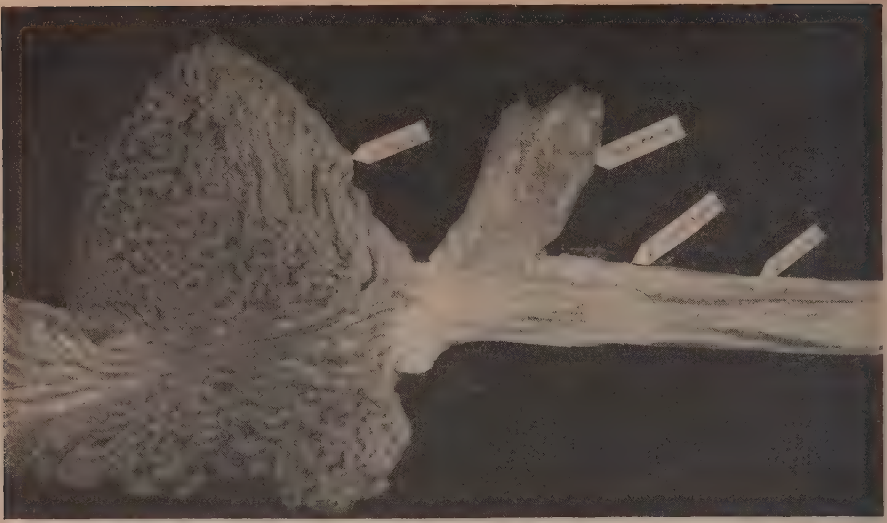

FIG. 1. Esophageal erosions produced by the diversion of bile and pancreatic juice into the esophagus following duodeno-esophagostomy.

In the former, the esophagi were greenish-brown, almost black, in appearance with numerous erosions and areas of hemorrhage after periods of perfusion lasting 3 to 8 hours. In the latter, the only changes were reddening of areas of the mucosa and a few erosions. In the 7 cases in which mixed bile and pancreatic juice did not produce severe changes in the perfused esophagus, either the tryptic activity was below normal, or the pH of the solution was not optimal for tryptic digestion.[†] In those perfusions carried out with sodium taurocholate or sodium glycocholate the entire wall of the esophagus was necrotic. Free fluid which had diffused through the esophageal wall was always present in the mediastinum. Human bile caused the same degree of change in the cats' esophagus as dog bile unless the bile was obtained from a patient who had been on T-tube drainage for a long period of time, thus depleting the bile salts in the bile. Four experiments with such a bile failed to produce esophagitis in a single instance. Microscopically the esophagi showing the most severe changes stained poorly with H & E. There was marked edema of the esophageal wall and poor cellular detail. Erosions of the mucous membrane were pres-

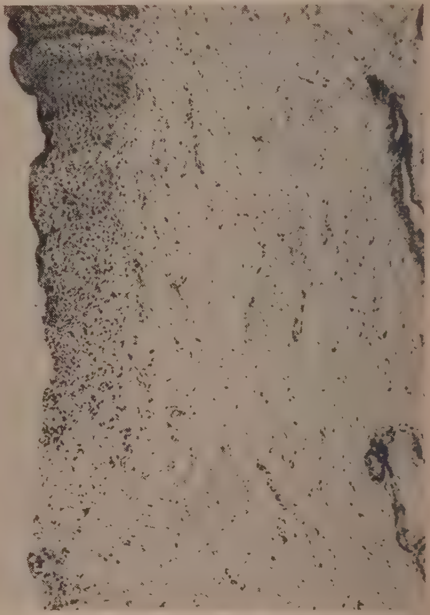

FIG. 2. Photomicrograph of an esophageal erosion caused by bile and pancreatic juice. Note that the lesion is shallow not extending beneath the muscularis mucosae.

[†] 0.2 cc of 1:10 dilution of test solution required to liquify 2 cc of gelatin substrate compared to the normal of 1.0 cc of a 1:100 solution. pH of test solutions ranged from 7.05 to 7.85. Optimal pH for tryptic digestion is 8-9.

TABLE II. Production of Esophagitis in Dogs.

Procedure	Total dogs	Esophagitis	No esophagitis
1. Bile and pancreatic juice diverted into esophagus	6	6	0
2. Bile diverted into esophagus	6	6	0
3. Pancreatic juice diverted into esophagus	6	6	0
4. Resection of acid secreting portion of stomach with diversion of bile and pancreatic juice into esophagus	4	4	0
5. Total gastrectomy with esophago-duodenostomy (regurgitation of bile and pancreatic juice)	7	5	2*
6. Excision of acid secreting portion of stomach; antral-esophageal anastomosis; extramucosal pyloroplasty	6	0	6
7. Succus entericus diverted into esophagus	5	0	5

* Bile duct occluded in one case not developing esophagitis.

ent with inflammatory cell infiltration of the submucosa.

B. Dogs. Bile, pancreatic juice, and mixtures of the two caused chronic lesions remarkably similar to each other when diverted into the esophagus of living dogs over periods of one to 3 months. At the time of esophagoscopy, as early as 8 days after the original operation, bleeding erosions were seen in the esophagus, and the mucosa was very easily traumatized. In these dogs there was a concurrent development of a severe anemia, either hypochromic or normochromic in nature, accompanied by positive benzidine and guaiac tests on the stools. The hemoglobin dropped to an average value of 9.5 g in the course of 4 to 8 weeks. The lowest value reached was 6.3 g, and in 4 dogs values between 7.0 and 8.0 g were observed. In order to keep these latter dogs alive, multiple transfusions were given. The erosions occurred over wide areas of the esophagus, most abundantly in the middle third, and often corresponded to the longitudinal folds of the mucous membrane (Fig. 1). Microscopically (Fig. 2) the lesions did not extend below the muscularis mucosae except in 2 instances when definite ulcers were present. Again, intestinal juice alone did not cause any changes nor was anemia observed.

It was also found that there was enough regurgitation of bile and pancreatic juice when an end to end esophago-duodenostomy was done to cause esophagitis in 5 out of 7 dogs. This was not true when the antrum remained as a reservoir even when an extramucosal pyloroplasty was done. When the entire acid secreting portion of the stomach was excised in addition to diverting all the bile and pancreatic juice into the esophagus, the development of esophagitis and anemia was observed consistently (Table II, item 4). In fact, the esophagitis and the anemia developing under these circumstances were comparable in every way to that observed when the stomach was intact. This finding rules out the regurgitation of acid gastric juice as being a factor in the development of the esophagitis. These results are summarized in Table II.

Discussion. The ability of bile and pancreatic juice to cause erosions of the esophagus has been established, and it has been shown that there is enough regurgitation of these juices after total gastrectomy and subsequent duodeno-esophagostomy in the dog to produce esophagitis. These observations confirm the suggestion that, when a total gastrectomy is done in man, a technic should be employed that precludes the regurgitation of the contents of the duodenal loop into the esophagus. Utilization of the Roux Y-plasty should be the best means to avoid this occurrence. In 2 dogs, erosions and hemorrhagic areas in the stomach, as well as esophagitis, were observed when the bile alone was diverted into the esophagus. These observations suggest the possibility that occult bleeding following gastric resection for duodenal ulcer in man may have its origin in the partial regurgitation of bile and pancreatic juice into the stomach through the gastrojejunostomy stoma. That esophagitis, of whatever origin in man, may be the source of some occult anemias appears likely.

Conclusions. (1) Digestion and destruction of the living esophageal mucosa in cats and dogs can be accomplished by bile, pancreatic juice, and mixtures of the two. (2) Bleeding from esophageal erosions attending the complete diversion of bile and pancreatic juice into the esophagus in the dog causes a severe secondary anemia. (3) The development of the esophagitis and anemia in the dog is not altered by total excision of the acid secreting portion of the stomach.

Received June 19, 1951. P.S.E.B.M., 1951, v77.

Effect of Inhaled Cigarette Smoke on Production of Peptic Ulcer in the Dog.* (18951)

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The effect of tobacco smoking on the etiology and course of peptic ulceration in man remains obscure. There is no reliable evidence that tobacco smoking causes peptic ulceration either experimentally or clinically. Barnett(1) failed to find a significantly greater proportion of smokers in 500 cases of peptic ulcer and gastric neurosis than in the general population. Gray(2) found 20% of ulcer patients failed to improve on medical management until tobacco was withdrawn. Batterman and Ehrenfeld(3) reported medical failures in 53.2% of habitual smokers and in 15% of non-smokers. Of the smokers who stopped of their own accord all responded satisfactorily to therapy; however when smoking was resumed, 85% had almost immediate recurrence of their disease. Trowell(4) found that men suffering from duodenal ulcer do not, on the average, smoke more tobacco than normal man but the practice of inhaling is twice as common in the ulcer group. Wolf and Wolff(5) reported no alteration in the motor and secretory activity of the stomach and color of the mucosa by pleasurable smoking. The purpose of this investigation was to study the effect of inhaled cigarette smoke on the production of experimental peptic ulceration in the intact dog.

Method of study. Healthy adult mongrel dogs were used in all experiments. All dogs had been under observation for at least one month and were free of distemper. Permanent lateral tracheostomies were prepared in the following manner: Through a vertical midline incision in the neck the trachea was dissected free and delivered into the wound. The strap muscles of the neck were sutured together beneath the trachea to form a sling-like support. The anterior one-third of 3 tracheal rings was excised, leaving the underlying tracheal mucous membrane intact. From a central point in this square window, radial incisions were made in the tracheal membrane to the 4 corners of the window. This resulted in 4 triangular flaps of tracheal mucosa. The edges of the skin incision were sutured to the edges of the tracheal window so that the sutures passed through the tracheal cartilage and the mucosal flaps were approximated to the raw skin edges. After a recovery period of at least one month, the tracheostomy was fitted with an ordinary T-tube and a small cigarette holder was adapted to the tube. Lighted cigarettes were placed in the holder and the dogs were allowed to inhale the smoke directly into the bronchial tree. Inhalation

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* These researches received support from the following sources: 1. The United States Public Health Service (Ulcer Project), Contract RG1026-C4; 2. The Augustus L. Searle Fund; 3. The Austen S. Cargill Fund; and 4. The Graduate School of the University of Minnesota.

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3. Batterman, R. C., and Ehrenfeld, I., *Gastroenterology*, 1949, v12, 575.

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5. Wolf, S., and Wolff, H. G., Human Gastric Function, Oxford University Press, New York, 1943.

was intermittent to correspond to the human smoker. All animals tolerated this procedure well and seemed to suffer no ill effects. The daily number of cigarettes was administered over a 4-hour period. A daily intramuscular injection of histamine in beeswax was given in doses corresponding to 30 mg of base, according to the method of Code and Varco(6). At the time of injection, food pans were removed until the following morning. All animals, including controls, followed the same schedule of feeding, histamine injections, and time spent in the smoking room. All animals were fed a stock diet of vitamin enriched dog biscuit kibbles supplemented by daily fresh horse meat and milk.

Results. Series I. Effect of cigarette smoke alone. Five dogs were allowed to smoke 5 cigarettes daily for 6 months. When sacrificed there was no evidence of gastrointestinal ulceration or erosion. All animals remained in excellent health and showed a weight gain of 6 to 10 lb. Gross examination of the bronchial tree, lungs and coronary arteries showed no pathological changes.

Series II. Effect of histamine alone. A. In unoperated dogs. Ten dogs received a daily injection of histamine for 5 days and were then sacrificed. No lesions of the gastrointestinal tract were found. *B.* In tracheostomized dogs. A second series of 5 dogs had a lateral tracheostomy performed, and after a 30-day recovery period were given daily injections of histamine for 5 days. No ulcers or erosions were found in this group when sacrificed. All dogs showed a weight loss of one to 3 lb. In previous papers reported from this laboratory, the observed incidence of peptic ulcer following the daily administration of 30 mg of histamine base to healthy normal dogs over a 5-day period has varied from 10 to 20%. The longer observation period to allow the dogs to become completely adjusted to the laboratory environment may account for the complete absence of peptic ulceration in this series.

Series III. Effect of cigarette smoke and histamine. A. Thirty days or more after lateral tracheostomy, 5 dogs were subjected to the smoke of 5 cigarettes and intramuscular injections of histamine daily for 5 days. Peptic ulceration of the duodenum was found in 2 dogs and a third showed a small superficial duodenal erosion. Two dogs showed no changes in the gastrointestinal tract. No perforation occurred in this group. All animals showed a weight loss of one to 6 lb. *B.* This series was similar to the preceding group except that the daily dose of inhaled smoke was doubled. Five dogs received the smoke of 10 cigarettes daily plus daily injections of histamine for 5 days. Peptic erosion or ulceration occurred in all animals. Duodenal erosion was found in 2, duodenal ulceration in 2, and both gastric and duodenal ulcers in one. There were no perforations. Four animals showed a weight loss of 1½ to 2½ lb and one gained 1½ lb. *C.* To test the thesis that the animals might develop a protective tolerance to smoke, 5 dogs received the smoke of 5 cigarettes daily for 30 days and then received both smoke and histamine for 5 days. In this group 2 animals were negative when sacrificed. One animal was found to have a duodenal erosion and 2 had multiple duodenal ulcerations, one of which was perforated (Fig. 1). In the animals with ulceration, a weight loss of 3 to 6 lb occurred during the smoke-histamine treatment. The other animals either maintained their weight or showed a weight gain.

Series IV. To determine how long dogs could survive the simultaneous administration of cigarette smoke and histamine, 5 animals received 5 cigarettes and histamine daily until death. Three dogs died in 5 to 12 days of typical chronic duodenal ulcers with perforation. One animal died of severe hemorrhage from a non-perforated duodenal ulcer on the 14th day. The remaining dog died of an acute gastric ulcer with perforation on the 51st day. The animals with duodenal ulcers all had a weight loss of 2 to 9 lb. The animal with gastric ulcer gained 2 lb before death.

Series V. The effect of "denicotinized" cigarettes ('SANO') was studied in a group of 5 dogs that received 10 such cigarettes daily plus histamine daily for 5 days and were then

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6. Code, C. F., and Varco, R. L., PROC. SOC. EXP. BIOL. AND MED., 1940, v44, 475.

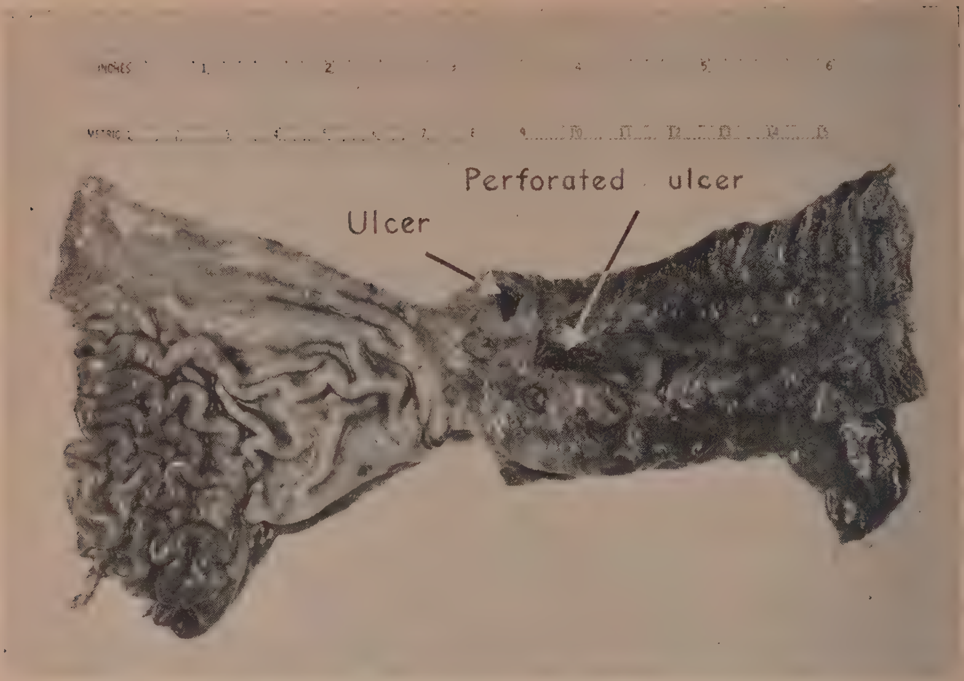

FIG. 1. Series III C, dog No. 4—Multiple duodenal ulcers with one perforation.

sacrificed. Duodenal ulceration without perforation occurred in one animal. The gastrointestinal tract of the remaining 4 dogs was negative. All sustained a weight loss of one to 5 lb.

Series VI. The effect of cigarette smoke inhalation on gastric secretory response was studied in 4 dogs with tracheostomies and total gastric pouches. In 16 trials there was either no effect or a slight decrease in volume and free acidity as compared to the standard fasting response for each dog. Schnedorf and Ivy(7) have reported similar results with Pavlov pouches.

Discussion. (1) Spontaneous peptic ulceration, rarely if ever, occurs in the intact dog. The daily inhalation of cigarette smoke over a period of 6 months failed to produce any gastrointestinal lesions in dogs. The animals all ate well during the experiment and either maintained their weight or showed an actual weight increase. After the first day or two the animals exhibited no signs of irritation and remained very quiet during the inhalation of smoke. This was true only if the inhalation was intermittent; *i.e.,* alternated with breathing pure air in the manner of human smokers. Continuous inhalation of smoke led to prostration, and if allowed to proceed, the animal would die. Autopsy usually revealed pulmonary edema. (2) The ulcerogenic effect of histamine in beeswax on the dog's gastrointestinal tract has been well documented(8). In intact healthy animals the incidence is quite low when administered over a short time interval. In this series of unoperated dogs or 30 days after lateral tracheostomy, no ulcerations or erosions occurred over a 5-day interval. (3) The abetting factor of cigarette smoke was tested in dogs receiving histamine in beeswax. Of 15 dogs so tested 11 or 73% developed gastroduodenal ulcerations or erosions in 5 days. No ulcers or erosions were

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7. Schnedorf, J. G., and Ivy, A. C., *J.A.M.A.,* 1939, v112, 898.

8. Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., *Surg., Gynec. and Obst.,* 1942, v75, 170.

found in 15 dogs on histamine alone. When the number of cigarettes was doubled the incidence of peptic ulcers or erosions increased from 60% to 100%. Under identical conditions denicotinized cigarettes did not provoke as high an incidence of ulcer-erosion as was obtained with ordinary cigarettes. Exposure to cigarette smoke for 30 days prior to the administration of histamine afforded little protection to the development of gastrointestinal lesions. In 5 dogs the continued simultaneous administration of cigarette smoke and histamine was uniformly fatal in 51 days. Four died of perforation and one of massive hemorrhage. According to Hay *et al.*(8) the daily injection of histamine to fresh unselected stock dogs produced ulceration in 87.5% in 37 days. About 1/3 of these were perforated. The secretory response of total gastric pouches in dogs was either unaffected or slightly decreased by the inhalation of cigarette smoke.

Conclusion. (1) The daily inhalation of cigarette smoke failed to produce peptic ulcers in dogs. (2) Cigarette smoke abets the ulcerogenic effect of histamine in beeswax; this is believed to be a nicotine effect. (3) Cigarette smoke does not stimulate gastric secretion in dogs.

Received June 19, 1951. P.S.E.B.M., 1951, v77.

Transplantability of Rabbit Ovaries in the Anterior Chamber of the Rabbit Eye.* (18952)

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(Introduced by C. A. Handley.)
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Survival and growth of autologous, homologous and heterologous tissue transplants in the anterior chamber of the eye has been observed and confirmed by many investigators(1-7). By the use of this method, Goodman(4) transplanted immature rat ovaries into 60 female and 20 male rats and found the percentage of takes in non-castrated males and non-spayed females, 2 months after transfer, to be 90% and 89%, respectively. Takes of transplants in females spayed concurrently and in those spayed 2 months after transfer were 100% and 33%, respectively, when examined 6 months after this operation. Continued growth of transplants in normal males castrated 2 months after transfer was evident in 80% of this group. Goodman utilized a Graefe knife for the corneo-scleral incision and curved, smooth, iris forceps to implant the tissue into the anterior chamber. Dunham, Watts, and Adair(6) studied newborn and adult rat ovaries transplanted into the anterior chambers of 137 rats. The percentage of takes was 74% in castrated males and 72% in spayed females, while those in non-castrate males and non-spayed females were 47% and 40%, respectively. A further group of spayed females and castrated males which had received chorionic gonadotrophin 3-14 days prior to tissue transfer yielded the highest percentage of takes (83%). The lowest percentage of takes was found when adult ovarian tissue was used. These workers used an angular keratome to make the ocular incision and donor tissue was inserted with a

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* Aided by grants to the General Research Funds, Baylor University College of Medicine, Houston 5, Texas by the M. D. Anderson Foundation, and by U. S. Public Health Service Cancer Teaching grant 692-C-2.

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3. Markee, J. E., *Am. J. Physiol.*, 1932, v100, 32.
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5. Goodman, L., *Anat. Rec.*, 1934, v59, 223.
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TABLE I. Autologous Ovarian Transplants into Anterior Chamber of Rabbit Eyes.

	4-wk donors		8-wk donors		12-wk donors		All age donors	
	Grafts	Takes	Grafts	Takes	Grafts	Takes	Grafts	Takes
Spayed	4	3	4	3	4	3	12	9 (75%)
Non-spayed	4	3	4	4	4	3	12	10 (83%)

TABLE II. Homologous Ovarian Transplants into Anterior Chamber of Rabbit Eyes.

	Newborn donors		4-wk donors		8-wk donors		12-wk donors		All age donors	
	Grafts	Takes	Grafts	Takes	Grafts	Takes	Grafts	Takes	Grafts	Takes
Castrate males	8	4	8	7	8	8	8	7	32	26 (81%)
Non-castrate males	8	7	8	3	8	5	8	7	32	22 (69%)
Spayed females	—	—	4	2	4	3	4	4	12	9 (75%)
Non-spayed females			4	4	4	4	4	3	12	11 (92%)

modified 19-gauge spinal needle.

It has been suggested by Robertson(8) in accordance with Halsted's(9) law of "endocrine deficiency" that an ovarian endocrine deficiency facilitates successful transfer of homologous ovaries into a new host.

The purpose of the present investigation is to reevaluate the transplantability of autologous and homologous young rabbit ovarian tissue. The donor tissue was removed from rabbits of different age groups.

Materials and methods. Sixty-six New Zealand albino and 2 Dutch rabbits of known age were studied in 4 groups: spayed females, non-spayed females, castrate males, and non-castrate males. Castrated male rabbits were allowed several weeks for recovery from this operation before transplants were made. Autologous transfer was made into the right eye and homologous transfer into the left eye of all females; those exposed to a unilateral oophorectomy were considered "non-spayed." Homologous transfers in the females were made at the time of the recipient's uni- or bilateral oophorectomy or on the following day. In the majority of males both eyes were used.

Transplantation of grafts was made under sterile conditions into all animals within 10-15 minutes after the removal of the ovary. The technic of transplantation used was that described by Greene(7), with the following modification: 15,000 units of penicillin G were given intramuscularly immediately after transfer. All animals were killed by injection of air into the heart. Microscopic sections of all the transplants were fixed in formalin, cut at 7 microns, stained with hematoxylin and eosin, and examined histologically.

Results. 114 transplants were made; 2 of these were not included in the results due to death of the recipients the day following transfer. Transplantation data are presented in Tables I and II. Criteria of growth were the presence of vascularity, "rounding off," and increase in size of the fragments, together with histologic evidence of developing follicles and ovarian stroma. In those animals in which growth did not occur, the transplanted fragments slowly decreased in size, and became dense white with no evidence of vascular response and ultimately became tiny scars. The gross assessment of the presence or absence of infection was not reliable. Inflammatory reaction was microscopically demonstrable in 19 cases, of which 16 were successful takes.

Hemorrhagic follicles developed in grafts in 2 non-spayed females within 24 hours after copulation. Glaucoma occurred in 4 grafts (3%); follicle cysts developed in 5 others.

Discussion. The incidence of homologous takes was highest in non-spayed females,

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8. Robertson, G. G., PROC. SOC. EXP. BIOL. AND MED., 1945, v59, 30.

9. Halsted, W. S., *J. Exp. Med.*, 1909, v11, 175.

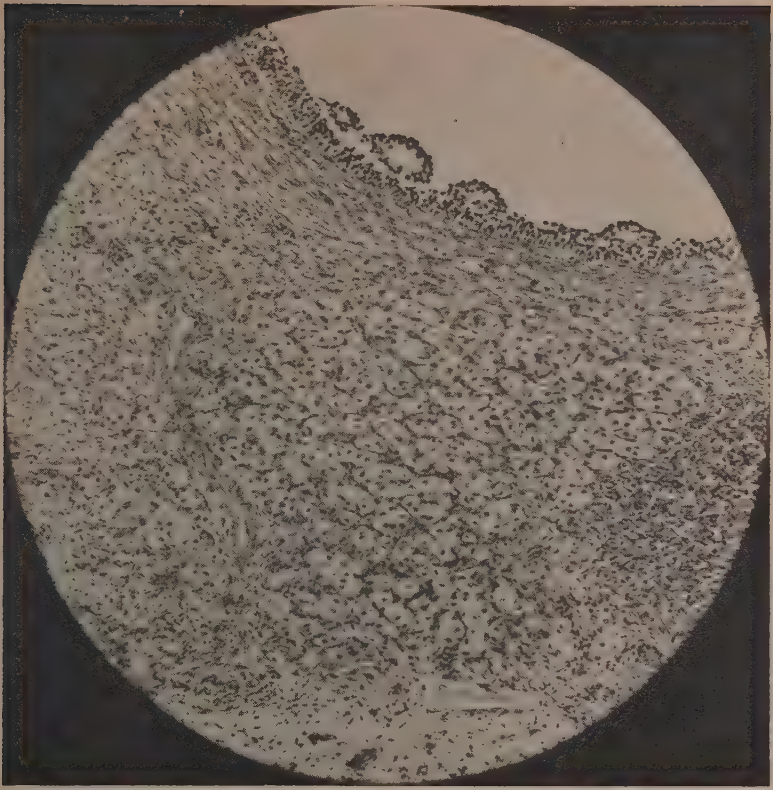

FIG. 1. Viable ovarian graft exhibiting an area of luteinization and a portion of a cystic follicle. Mg. × 115.

92%, with takes of 81% and 75% in castrate males and spayed females, respectively. Since none of the grafted ovarian tissues were mature as evidenced by histological examination, our work does not refute Halsted's theory(9). The number of takes in the group of non-castrate males was 69%. It was found that a higher number of autologous takes occurred in non-spayed females, 83%. The best results were achieved in 12-week- and 8-week-old tissue grafts and the poorest in newborn and 4-week. Little difference was found between the overall homologous and autologous female graft takes.

The average weight curve of all animals with viable transplants corresponds closely with a normal weight curve of a control group of non-transplanted animals, thereby giving further support to the supposition of Hill(10) that functioning ovarian grafts in the ear did not influence weight gain.

The technic of transfer utilized by the investigators cited above when compared with that used in the present experiment, did not appear to appreciably influence transplantability.

Summary. In 112 autologous and homologous anterior chamber grafts of ovarian tissue in castrate and non-castrate males, and spayed and non-spayed females, the percentage of homologous takes was highest in non-spayed females, next highest in castrate males and spayed females. The lowest percentage of such takes was in normal males. The incidence in the different categories is not considered to be statistically significant with the

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10. Hill, R. T., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 313.

possible exception of the non-spayed group of females. The ages of the donor grafts were not significant in relation to growth of fragments at the ages investigated. There was no apparent influence of the tissue transplants upon the weight gain of the recipient rabbits.

The authors wish to acknowledge the valuable assistance of Dr. Hebbel E. Hoff, Department of Physiology, B.U.C.M., and Dr. G. Gordon Robertson, Department of Anatomy, B.U.C.M.

Received June 29, 1951. P.S.E.B.M., 1951, v77.

Parenteral Nutrition. XI. Studies with Stable and Unstable Fat Emulsions Administered Intravenously.* (18953)

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It has been clearly demonstrated that suitably prepared emulsions of fat can be given safely in large quantities to various species of experimental animals(1,2), and to man(3). Furthermore, studies concerning nitrogen and potassium balances(3,4), rates of growth (1,4), and analyses of body fat(1,4), have demonstrated the utilization of the fat in such emulsions. Experiments with emulsified C^{14}-labeled trilaurin(5) and tripalmitin(6) have shown the metabolism of these lipids to be rapid and extensive. *In vitro* studies with emulsified C^{14}-labeled trilaurin have indicated that various rat tissues have a high capacity to metabolize this and other lipid substrates(7). Rat blood, moreover, has little effect on these substrates directly or in affecting the metabolism of these substances by tissue slices. Thus, the evidence supports the assumption that the blood plays a minor direct role, if any, in the utilization of intravenously administered emulsified fats.

The question arises, however, as to the ability of the body to remove extracellular fat which, for example, has become lodged in capillaries. This problem is of *no* concern when finely dispersed, stable emulsions of fat are employed. It *is* of great concern when an emulsion is administered intravenously which (a) is not finely dispersed, but contains large particles of fat (3 to 15 μ), (b) is finely dispersed, but breaks down when in contact with plasma, or (c) is finely dispersed, but breaks down because of concurrent infusions of materials not compatible with the emulsified fat. Studies with iodinated fat emulsions of both a high and low degree of dispersion have been reported(8). The data presented in this paper deal with both stable and unstable emulsions which were studied from two standpoints: (1) the increase in the fat content of various tissues following the administration of such emulsions and (2) the rate of removal from the tissues of the excess fat which resulted when these emulsions were given.

Experimental. A number of emulsions of

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* Supported in part by grants-in-aid from The Upjohn Company, Kalamazoo, Mich., The Nutrition Foundation, Inc., New York City and The Cancer Research Grants Branch, National Cancer Institute, Bethesda, Md.

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TABLE I. Composition of Emulsions.*

Emulsion No.	Corn oil,† %	Coconut oil,‡ %	Stabilizer system B(F^2)§	Stabilizer system B(F^1)§	Stability *in vitro*
1	—	30	3	—	Stable
2	30	—	3	—	,,
3	30	—	—	3	Unstable
4	30	—	—	1	,,

* All emulsions were made with 5% dextrose solution as the aqueous phase. They were all stable to autoclaving.

† Refined corn oil. Corn Products Refining Co., Argo, Ill.

‡ Edible coconut oil. E. F. Drew and Co., Boonton, N. J.

§ Prepared from purified soybean phosphatides(11).

coconut and corn oils were prepared by the high-pressure homogenization procedure previously described(9). Sterilization of all emulsions was accomplished by autoclaving at 15 lb per square inch for 15 minutes. Pertinent data including the composition of these emulsions are given in Table I. It should be emphasized that all of the emulsions used in these studies contained 30% fat, not 15% as is usual in the emulsions used clinically by this laboratory. The stability of the emulsions in the presence of plasma was tested as follows: 0.5 ml of emulsion was mixed with 2 ml of heparinized dog plasma and the mixture was incubated at 37.5°C for 4 to 6 hours. Samples of the mixture were removed at various time intervals and examined under the microscope for evidence of a change in the size of the particles of fat. Other samples were simultaneously removed, diluted to 2 ml with 5% dextrose and the turbidity of the mixture was determined by means of the Klett-Summerson photoelectric colorimeter as described previously(10). Emulsions No. 1 and 2 were found to remain stable, whereas No. 3 and 4 slowly broke down to larger fat particles.

Non-fasted male rats of the Wistar strain, weighing approximately 200 g, were used in all experiments and were maintained on a commercial stock feed. Injection of the emulsions was done through the tail vein while the animal was under light ether anesthesia. The quantity of emulsified fat administered varied between 2.5 and 10 g of fat per kilo body weight. Some animals were sacrificed when the level of infused fat remaining in the blood was negligible as determined by turbidimetric analysis. The remaining animals were sacrificed at various intervals after the blood fat level had returned to normal. In several instances the animals died and their tissues were used since the time of death was fairly well known. Animals which had received injections of 5% dextrose solution were also sacrificed and their organs were analyzed for the normal fat content. Histological sections were made to confirm the location of the fat in the tissues.

Fat analysis of the lung, liver, brain, spleen, and kidney was done as follows: the fresh tissue was weighed, a suitable sample of it was ground in a small mortar with powdered anhydrous Na_2SO_4, and the mixture was extracted with hot $CHCl_3$ in a continuous extractor for 8 hours. The chloroform solution of lipids was then evaporated to dryness; traces of solvent being removed by means of a stream of nitrogen. The residue was repeatedly extracted with small portions of hot petroleum ether (B.P. 65°C) and all of the extracts were filtered through a sintered glass funnel into a tared 50 ml conical flask. The funnel was washed with small quantities of petroleum ether and the washes were also collected in the flasks. After complete removal of the ether, the flasks were placed in a desiccator for several hours and then weighed. The percent of fat was calculated from the weight of the extracted ether-soluble lipids.

Results and discussion. As judged by gross

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TABLE II. Fat Content of Various Tissues After Injection of Emulsions.*

Group No.	No. of animals	Body wt	Emulsion No.	g fat/kg body wt	% fat (wet basis)				
					Spleen	Liver	Lung	Brain	Kidney
I	3	236 (219–257)	5% dextrose	0	2.5 (2.3– 2.6)	4.5 (4.5– 4.5)	3.9 (3.8– 4.1)	7.8 (7.3– 8.3)	4.2 (3.8–4.5)
II	3	162 (160–167)	1	5	3.2 (2.4– 4.5)	6.5 (6.2– 6.7)	4.7 (4.4– 5.1)	8.8 (8.6– 8.9)	4.4 (4.2–4.5)
III	3	228 (208–245)	2	5	5.7 (4 – 7.2)	6.9 (6.7– 6.9)	3.8 (3.1– 4)	9.4 (9.1– 9.6)	3.4 (3.2–3.6)
IV	3	228 (208–245)	3	5	30.5 (28.2–32.1)	6.4 (6 – 6.5)	6.1 (4.6– 9)	9.8 (8.6– 9.9)	4.6 (4.3–4.9)
V	3	231 (205–246)	3	10	26.3 (21.4–33.1)	8.1 (6.6– 8.8)	10 (8 –13.1)	9.8 (9.6–10)	5.2 (5 –5.4)
VI	3	259 (241–286)	4	10	34.5 (24 –42.8)	11.5 (9.2–14.9)	20.3 (14.1–25.2)	10.5 (8.4–13.1)	5.6 (4.1–7.1)

* Animals sacrificed when blood level had returned to normal.

TABLE III. Effect of Dosage of Fat Emulsions on the Fat Content of Various Tissues.*

Group No.	No. of animals	Body wt	Emulsion No.	g fat/kg body wt	% fat (wet basis)				
					Spleen	Liver	Lung	Brain	Kidney
VII	3	171 (166–177)	1	2.5	2.9 (2.5– 3.3)	5.9 (4.7–6.7)	4.7 (4.3– 5.1)	8.8 (8.1– 9.2)	4.3 (4.1–4.3)
II	3	162 (160–167)	1	5	3.2 (2.4– 4.6)	6.5 (6.2–6.7)	4.7 (4.4– 5.1)	8.8 (8.6– 8.9)	4.4 (4.2–4.5)
VIII	3	216 (205–222)	3	2.5	15.9 (14.8–17)	5.6 (5 –6.5)	4.2 (3.9– 4.9)	9 (8.9– 9.1)	4.9 (4.6–5.2)
IV	3	193 (190–200)	3	5	30.5 (28.2–32.1)	6.4 (5.9–6.6)	6.1 (4.6– 9)	9.8 (8.6– 9.9)	4.7 (4.3–4.9)
V	3	231 (205–246)	3	10	26.3 (24.4–33.1)	8.1 (6.6–8.9)	10 (8 –13.1)	9.8 (9.6–10)	5.2 (4.9–5.4)

* Animals sacrificed when blood fat level had returned to normal.

fat analysis, the fat content of various rat organs increased moderately following the intravenous injection of stable, finely dispersed emulsions of fat. On the other hand, a fairly great increase in fat content occurred when the emulsions used were finely dispersed, but unstable *in vivo*. These phenomena are demonstrated by the data presented in Table II. All of the emulsions (see Table I) were equivalent from the standpoint of size of the fat particles as determined by visual microscopy employing an ocular micrometer. Emulsions No. 1 and 2 were stable *in vivo* in the presence of blood; whereas, Emulsions No. 3 and 4 were unstable *in vivo* presumably because of the effect of the electrolytes contained in the plasma. The latter emulsions were also unstable *in vitro* when mixed with heparinized rat plasma at 38°C. The breakdown of these emulsions either *in vivo* or *in vitro* was not immediate and complete, but rather progressive over a period of several hours. This type of instability would enable fat to accumulate extracellularly in many tissues instead of lodging mainly in the first tissue through which it passed, which might be the case if immediate, complete breaking occurred. Histological sections of lung, liver, brain, spleen, and kidney showed so much extracellular fat present after the injection of unstable emulsions that it was impossible to discern the tissue structure.

The data in Table II show that the highest *relative* increase in fat content occurred in the spleen with both stable and unstable emulsions. The liver showed the next highest increase when stable emulsions were used, but the lung was second highest when the unstable emulsions were used. Thus, it would appear that an appreciable increase in fat content of the lungs following intravenous administration of fat would be one indication that the emulsion was unstable. Little increase in fat content of either brain or kidney tissue resulted after the administration of stable emulsions. A greater increase in the total lipid of these two tissues occurred after injection of the unstable emulsions, especially Emulsion No. 4.

As shown by the data in Table III, increasing the dose of the stable fat emulsion (Emulsion No. 1) from 2.5 g fat per kilo of body weight to 5.0 g did not alter very much the total content of the organs. In the case of the unstable emulsion (Emulsion No. 3) this increase in dose raised the fat content of the spleen from approximately 16% up to 30%. The slight increase in the case of the liver and lung is interesting. When the dose of Emulsion No. 3 was raised to 10 g of fat per kilo of body weight, an increased fat content resulted in all organs analyzed. It should be pointed out that at the lowest dose used, the total-fat analysis method used here is not delicate enough to indicate very small changes in fat content. A more exact approach to this problem is available through the use of emulsions of radioactive triglycerides.

Data on the rate of removal of fat from the various organs after administration of fat emulsions are presented in Table IV. The rats in Groups IX, X, and XI which received a stable emulsion removed the excess fat from the various organs in fairly short time, for an almost normal level was reached in 24 hours. The amount of fat which these rats were given (5 g/kg body weight) is a much greater dose than would be given to patients requiring about 1500 to 2000 calories from a fat infusion. The animals which received Emulsion No. 4 were given an even higher dose per kg of body weight than those which received the stable emulsion (Emulsion No. 2). It is apparent that even under such drastic conditions the animals survived and the increased fat content of the organs slowly returned to normal. Some of this increase in fat content would be due to lipid which had gained access to the various cells prior to the complete breakdown of the emulsion. However, most of it was extracellular and was probably removed by phagocytic and lipolytic action. Whether a rise in serum lipase results after

TABLE IV. Rate of Removal of Fat from Various Organs After Administration of Stable and Unstable Emulsions.

Group No.	No. of animals	Body wt	Emulsion No.	g fat/kg body wt	Time sacrificed (min)	% fat (wet basis)				
						Spleen	Liver	Lung	Brain	Kidney
IX	3	291 (285–290)	2	5	290 (285–295)	9.3 (6.6–11.1)	7.4 (7.3– 7.4)	5.5 (5.5– 5.6)	9.9 (9.7–10.1)	5.6 (4.4–8.3)
X	3	206 (195–219)	2	5	1307 (1025–1460)	3.4 (3.2– 3.6)	6.5 (6.3– 6.8)	4.3 (3.4– 5)	8.8 (8.3– 9.2)	4.3 (3.5–5.4)
XI	3	208 (200–214)	2	5	4341 (4340–4345)	2.4 (2.2– 2.5)	5 (3.1– 6.1)	3.1 (2.1– 4.1)	9 (8.8– 9.1)	4 (3.5–4.5)
VI	3	259 (241–286)	4	10	165	34.5 (24 –42.8)	11.8 (9.2–14.9)	20.3 (14.1–25.2)	10.4 (8.4–13.1)	5.6 (4.1–7.1)
XII	3	236 (213–260)	4	10	710 (690–720)	22.6 (15.5–31.1)	9.8 (9 –11.1)	13.3 (11.6–17.6)	10 (8.9–11.5)	5.7 (4 –6.9)
XIII	3	234 (220–257)	4	10	5726 (5024–6160)	23.4 (20.2–34.7)	4.6 (3.5– 5.8)	9.6 (7.1–11.3)	8.8 (8.4– 9.2)	4 (3.5–4.4)
XIV	3	257 (255–260)	4	10	11463 (11160–11650)	13.6 (8.8–21.3)	5.3 (3.8– 7.9)	5 (4.7– 5.4)	9.4 (8.7– 9.9)	4.6 (3.7–5.5)

unstable emulsions are introduced into the blood stream remains to be determined.

Thus, it can be seen that from the standpoint of safety in the intravenous administration of fat emulsion there is little danger of embolic phenomena occurring, especially when stable, finely dispersed emulsions of fat are used.

Summary. Both stable and unstable emulsions of fat have been administered intravenously to rats. The stable emulsions caused a moderate increase in total lipids of the spleen, liver, and lungs; whereas, a very marked increase was observed when unstable emulsions were given. Much of the increase due to the latter type of emulsion was caused by large extracellular fat globules. The removal of the excess fat in the tissues has been studied and found to be rapid in the case of stable emulsions, and slower but definite in the case of the unstable emulsions.

We wish to express appreciation to Associated Concentrates, Inc., Elmhurst, L. I., N. Y., Corn Industries Research Foundation, New York City, and The Upjohn Co., Kalamazoo, Mich. for various materials used in this research.

Received October 19, 1950. P.S.E.B.M., 1951, v77.

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